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Bescheinigung | Certificate | Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

94200738.6

Der Präsident des Europäischen Patentamts:
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

D. RADFORD

Den Haag, den
The Hague, 23/03/95
La Haye, le

Eur pälsches Patentamt
Eur pean Patent Office
Office européen des brevets

Blatt 2 der B scheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.: 94200738.6
Demande n°:

Anmeldetag:
Date of filing: 22/03/94
Date de dépôt:

Anmelder:
Applicant(s):
Demandeur(s):
RIJKSUNIVERSITEIT TE UTRECHT
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NETHERLANDS

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
Pharmaceutical compostion for the treatment and prevention of inflammatory diseases and active components of such compositions

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

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SEQ ID No. 1

Met Ala Lys Thr Ile Ala Tyr Asp Glu Glu Ala Arg Arg Gly Leu
5 10 15

Glu Arg Gly Leu Asn Ala Leu Ala Asp Ala Val Lys Val Thr Leu
20 25 30

Gly Pro Lys Gly Arg Asn Val Val Leu Glu Lys Lys Trp Gly Ala
35 40 45

Pro Thr Ile Thr Asn Asp Gly Val Ser Ile Ala Lys Glu Ile Glu
50 55 60

Leu Glu Asp Pro Tyr Glu Lys Ile Gly Ala Glu Leu Val Lys Glu
65 70 75

Val Ala Lys Lys Thr Asp Asp Val Ala Gly Asp Gly Thr Thr Thr
80 85 90

Ala Thr Val Leu Ala Gln Ala Leu Val Arg Glu Gly Leu Arg Asn
95 100 105

Val Ala Ala Gly Ala Asn Pro Leu Gly Leu Lys Arg Gly Ile Glu
110 115 120

Lys Ala Val Glu Lys Val Thr Glu Thr Leu Leu Lys Gly Ala Lys
125 130 135

Glu Val Glu Thr Lys Glu Gln Ile Ala Ala Thr Ala Ala Ile Ser
140 145 150

Ala Gly Asp Gln Ser Ile Gly Asp Leu Ile Ala Glu Ala Met Asp
155 160 165

Lys Val Gly Asn Glu Gly Val Ile Thr Val Glu Glu Ser Asn Thr
170 175 180

Phe Gly Leu Gln Leu Glu Leu Thr Glu Gly Met Arg Phe Asp Lys
185 190 195

Gly Tyr Ile Ser Gly Tyr Phe Val Thr Asp Pro Glu Arg Gln Glu
200 205 210

Ala Val Leu Glu Asp Pro Tyr Ile Leu Leu Val Ser Ser Lys Val
215 220 225

Ser Thr Val Lys Asp Leu Leu Pro Leu Leu Glu Lys Val Ile Gly
230 235 240

Ala Gly Lys Pro Leu Leu Ile Ile Ala Glu Asp Val Glu Gly Glu
245 250 255

Ala Leu Ser Thr Leu Val Val Asn Lys Ile Arg Gly Thr Phe Lys
260 265 270

Ser Val Ala Val Lys Ala Pro Gly Phe Gly Asp Arg Arg Lys Ala
275 280 285

SEQ ID No. 1

Met Ala Lys Thr Ile Ala Tyr Asp Glu Glu Ala Arg Arg Gly Leu
5 10 15

Glu Arg Gly Leu Asn Ala Leu Ala Asp Ala Val Lys Val Thr Leu
20 25 30

Gly Pro Gly Lys Arg Asn Val Val Leu Glu Lys Lys Trp Gly Ala
35 40 45

Pro Thr Ile Thr Asn Asp Gly Val Ser Ile Ala Lys Glu Ile Glu
50 55 60

Leu Glu Asp Pro Tyr Glu Lys Ile Gly Ala Glu Leu Val Lys Glu
65 70 75

Val Ala Lys Lys Thr Asp Asp Val Ala Gly Asp Gly Thr Thr Thr
80 85 90

Ala Thr Val Leu Ala Gln Ala Leu Val Arg Glu Gly Leu Arg Asn
95 100 105

Val Ala Ala Gly Ala Asn Pro Leu Gly Leu Lys Arg Gly Ile Glu
110 115 120

Lys Ala Val Glu Lys Val Thr Glu Thr Leu Leu Lys Gly Ala Lys
125 130 135

Glu Val Glu Thr Lys Glu Gln Ile Ala Ala Thr Ala Ala Ile Ser
140 145 150

Ala Gly Asp Gln Ser Ile Gly Asp Leu Ile Ala Glu Ala Met Asp
155 160 165

Lys Val Gly Asn Glu Gly Val Ile Thr Val Glu Glu Ser Asn Thr
170 175 180

Phe Gly Leu Gln Leu Glu Leu Thr Glu Gly Met Arg Phe Asp Lys
185 190 195

Gly Tyr Ile Ser Gly Tyr Phe Val Thr Asp Pro Glu Arg Gln Glu
200 205 210

Ala Val Leu Glu Asp Pro Tyr Ile Leu Leu Val Ser Ser Lys Val
215 220 225

Ser Thr Val Lys Asp Leu Leu Pro Leu Leu Glu Lys Val Ile Gly
230 235 240

Ala Gly Lys Pro Leu Leu Ile Ile Ala Glu Asp Val Glu Gly Glu
245 250 255

Ala Leu Ser Thr Leu Val Val Asn Lys Ile Arg Gly Thr Phe Lys
260 265 270

Ser Val Ala Val Lys Ala Pro Gly Phe Gly Asp Arg Arg Lys Ala
275 280 285

Met Leu Gln Asp Met Ala Ile Leu Thr Gly Gly Gln Val Ile Ser
290 295 300

Glu Glu Val Gly Leu Thr Leu Glu Asn Ala Asp Leu Ser Leu Leu
305 310 315

Gly Lys Ala Arg Lys Val Val Val Thr Lys Asp Glu Thr Thr Ile
320 325 330

Val Glu Gly Ala Gly Asp Thr Asp Ala Ile Ala Gly Arg Val Ala
335 340 345

Gln Ile Arg Gln Glu Ile Glu Asn Ser Asp Ser Asp Tyr Asp Arg
350 355 360

Glu Lys Leu Gln Glu Arg Leu Ala Lys Leu Ala Gly Gly Val Ala
365 370 375

Val Ile Lys Ala Gly Ala Ala Thr Glu Val Glu Leu Lys Glu Arg
380 385 390

Lys His Arg Ile Glu Asp Ala Val Arg Asn Ala Lys Ala Ala Val
395 400 405

Glu Glu Gly Ile Val Ala Gly Gly Gly Val Thr Leu Leu Gln Ala
410 415 420

Ala Pro Thr Leu Asp Glu Leu Lys Leu Glu Gly Asp Glu Ala Thr
425 430 435

Gly Ala Asn Ile Val Lys Val Ala Leu Glu Ala Pro Leu Lys Gln
440 445 450

Ile Ala Phe Asn Ser Gly Leu Glu Pro Gly Val Val Ala Glu Lys
455 460 465

Val Arg Asn Leu Pro Ala Gly His Gly Leu Asn Ala Gln Thr Gly
470 475 480

Val Tyr Glu Asp Leu Leu Ala Ala Gly Val Ala Asp Pro Val Lys
485 490 495

Val Thr Arg Ser Ala Leu Gln Asn Ala Ala Ser Ile Ala Gly Leu
500 505 510

Phe Leu Thr Thr Glu Ala Val Val Ala Asp Lys Pro Glu Lys Glu
515 520 525

Lys Ala Ser Val Pro Gly Gly Gly Asp Met Gly Gly Met Asp Phe
530 535 540

SEQ. ID No. 2

The alignment was done on 4 Protein sequences.
Character to show that a position in the alignment is perfectly conserved: '*'
Character to show that a position is well conserved: '.'

Alignment

```
 0$HUMAN   MLRLPTVFRQMRPVSRVLAPHLTRAYAKDVKFGADARALMLQGVDLLADA   50
  0$RAT    ------------------A--------KDVKFGADARALMLQGVDLLADA   24
 60$MOUSE  ------------------APHLTRAYAKDVKFGADARALMLQGVDLLADA   32
MBAA       M------------------------AKTIAYDEEARRGLERGLNALADA   25
                                    *.. ....**  . .*.. ****

P60$HUMAN  VAVTMGPKGRTVIIEQSWGSPKVTKDGVTVAKSIDLKDKYKNIGAKLVQD  100
P60$RAT    VAVTMGPKGRTVIIEQSWGSPKVTKDGVTVAKSIDLKDKYKNIGAKLVQD   74
P60$MOUSE  VAVTMGPKGRTVIIEQSWGSPKVTKDGVTVAKSIDLKDKYKNIGAKLVQD   82
MBAA       VKVTLGPKGRNVVLEKKWGAPTITNDGVSIAKEIELEDPYEKIGAELVKE   75
           * **.*****.*..*..**.*..*.***..**.*.*.* *..***.**..

P60$HUMAN  VANNTNEEAGDGTTTATVLARSIAKEGFEKISKGANPVEIRRGVMLAVDA  150
P60$RAT    VANNTNEEAGDGTTTATVLARSIAKEGFEKISKGANPVEIRRGVMLAVDA  124
P60$MOUSE  VANNTNEEAGDGTTTATVLARSIAKEGFEKISKGANPVEIRRGVMLAVDA  132
MBAA       VAKKTDDVAGDGTTTATVLAQALVREGLRNVAAGANPLGLKRGIEKAVEK  125
           **..*.. ************.....**. ... ****....**.  **.

P60$HUMAN  VIAELKKQSKPVTTPEEIAQVATISANGDKEIGNIISDAMKKVGRKGVIT  200
 60$RAT   VIAELKKQSKPVTTPEEIAQVATISANGDKDIGNIISDAMKKVGRKGVIT  174
  0$MOUSE  VIAELKKQSKPVTTPEEIAQVATISANGDKDIGNIISDAMKKVGRKGVIT  182
 BAA       VTETLLKGAKEVETKEQIAATAAISA-GDQSIGDLIAEAMDKVGNEGVIT  174
           *...* * .* *.* *.**..*.*** **..**..*..**.***..****

P60$HUMAN  VKDGKTLNDELEIIEGMKFDRGYISPYFINTSKGQKCEFQDAYVLLSEKK  250
P60$RAT    VKDGKTLNDELEIIEGMKFDRGYISPYFINTSKGQKCEFQDAYVLLSEKK  224
P60$MOUSE  VKDGKTLNDELEIIEGMKFDRGYISPYFINTSKGQKCEFQDAYVLLSEKK  232
MBAA       VEESNTFGLQLELTEGMRFDKGYISGYFVTDPERQEAVLEDPYILLVSSK  224
           *....*.. .**..***.**.**** **..... *.  ..*.*.** ..*
```

```
P60$HUMAN    ISSIQSIVPALEIANAHRKPLVIIAEDVDGEALSTLVLNRLKVGLQVVAV   300
P60$RAT      ISSVQSIVPALEIANAHRKPLVIIAEDVDGEALSTLVLNRLKVGLQVVAV   274
P60$MOUSE    FSSVQSIVPALEIANAHRKPLVIIAEDVDGEALSTLVLNRLKVGLQVVAV   282
MBAA         VSTVKDLLPLLEKVIGAGKPLLIIAEDVEGEALSTLVVNKIRGTFKSVAV   274
              *......* ** . .  ***.******.********.*... ... ***

P60$HUMAN    KAPGFGDNRKNQLKDMAIATGGAVFGEEGLTLNLEDVQPHDLGKVGEVIV   350
P60$RAT      KAPGFGDNRKNQLKDMAIATGGAVFGEEGLNLNLEDVQAHDLGKVGEVIV   324
P60$MOUSE    KAPGFGDNRKNQLKDMAIATGGAVFGEEGLNLNLEDVQAHDLGKVGEVIV   332
MBAA         KAPGFGDRRKAMLQDMAILTGGQVISEE-VGLTLENADLSLLGKARKVVV   323
              *******.**. *.**** ***.*..** ..*.**...   ***. .*.*

P60$HUMAN    TKDDAMLLKGKGDKAQIEKRIQEIIEQLDVTTSEYEKEKLNERLAKLSDG   400
P60$RAT      TKDDAMLLKGKGDKAHIEKRIQEITEQLDITTSEYEKEKLNERLAKLSDG   374
P60$MOUSE    TKDDAMLLKGKGDKAHIEKRIQEITEQLDITTSEYEKEKLNERLAKLSDG   382
MBAA         TKDETTIVEGAGDTDAIAGRVAQIRQEIENSDSDYDREKLQERLAKLAGG   373
              ***.. ...* **.. *. *...* .... ..*.*..***.******..*

  0$HUMAN    VAVLKVGGTSDVEVNEKKDRVTDALNATRAAVEEGIVLGGGCALLRCIPA   450
  0$RAT      VAVLKVGGTSDVEVNEKKDRVTDALNATRAAVEEGIVLGGGCALLRCIPA   424
  0$MOUSE    VAVLKVGGTSDVEVNEKKDRVTDALNATRAAVEEGIVLGGGCALLRCIPA   432
MBAA         VAVIKAGAATEVELKERKHRIEDAVRNAKAAVEEGIVAGGGVTLLQAAPT   423
              ***.*.*....**..*.*.*..**.....******** *** .**.  *.

P60$HUMAN    LDSLTPANEDQKIGIEIIKRTLKIPAMTIAKNAGVEGSLIVEKIMQSSSE   500
P60$RAT      LDSLKPANEDQKIGIEIIKRALKIPAMTIAKNAGVEGSLIVEKILQSSSE   474
P60$MOUSE    LDSLKPANEDQKIGIEIIKRALKIPAMTIAKNAGVEGSLIVEKILQSSSE   482
MBAA         LDELK-LEGDEATGANIVKVALEAPLKQIAFNSGLEPGVVAEKVRNLPAG   472
              **.*.  ..*. .* .*.* .*. * . ** *.*.* ....**. . ...

P60$HUMAN    VGYDAMAGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLTTAEVVVTEIP   550
P60$RAT      VGYDAMLGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLTTAEAVVTEIP   524
P60$MOUSE    VGYDAMLGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLTTAEAVVTEIP   532
MBAA         HGLNAQTGVYEDLLAAGVADPVKVTRSALQNAASIAGLFLTTEAVVADKP   522
              * .*  * . .... *. **.**.*.** .**..*.*. *.*.**.. *

P60$HUMAN    KEEKDPGMGAMGGMGGGMGGGMF    573
  0$RAT      KEEKDPGMGAMGGMGGGMGGGMF    547
  0$MOUSE    KEEKDPGMGAMGGMGGGMGGGMF    555
MBAA         EKEKASVPG-----GGDMGGMDF    540
              ..**..  *     **.***  *

Consensus length:  573
Identity  : 254 ( 44.3%)
Similarity: 211 ( 36.8%)
```

SEQ ID No. 3

TRANSLATION OF NUCLEIC ACID SEQUENCE OF THE MYCOB. BOVIS BCG HSP65 GENE

```
     580           590           600           610           620
      |             |             |             |             |
ATG GCC AAG ACA ATT GCG TAC GAC GAA GAG GCC CGT CGC GGC CTC GAG CGG GGC
Met Ala Lys Thr Ile Ala Tyr Asp Glu Glu Ala Arg Arg Gly Leu Glu Arg Gly  18

630           640           650           660           670           680
 |             |             |             |             |             |
TTG AAC GCC CTC GCC GAT GCG GTA AAG GTG ACA TTG GGC CCC AAG GGC CGC AAC
Leu Asn Ala Leu Ala Asp Ala Val Lys Val Thr Leu Gly Pro Lys Gly Arg Asn  36

       690           700           710           720           730
        |             |             |             |             |
GTC GTC CTG GAA AAG AAG TGG GGT GCC CCC ACG ATC ACC AAC GAT GGT GTG TCC
Val Val Leu Glu Lys Lys Trp Gly Ala Pro Thr Ile Thr Asn Asp Gly Val Ser  54

  740           750           760           770           780           790
   |             |             |             |             |             |
ATC GCC AAG GAG ATC GAG CTG GAG GAG CTG GAG GAT CCG TAC GAG GCC GAG CTG
Ile Ala Lys Glu Ile Glu Leu Glu Glu Leu Glu Asp Pro Tyr Glu Ala Glu Leu  72

          800           810           820           830           840
           |             |             |             |             |
GTC AAA GAG GTA GCC AAG AAG ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG ACG
Val Lys Glu Val Ala Lys Lys Thr Asp Asp Val Ala Gly Asp Gly Thr Thr Thr  90

     850           860           870           880           890
      |             |             |             |             |
GCC ACC GTG CTG GCC CAG GCG TTG GTT CGC CAG GGC CTG CGC AAC GTC GCG GCC
Ala Thr Val Leu Ala Gln Ala Leu Val Arg Gln Gly Leu Arg Asn Val Ala Ala 108

900           910           920           930           940           950
 |             |             |             |             |             |
GGC GCC AAC CCG CTC GGT CTC AAA CGC GGC ATC GAA AAG GCC GTG GAG AAG GTC
Gly Ala Asn Pro Leu Gly Leu Lys Arg Gly Ile Glu Lys Ala Val Glu Lys Val 126

       960           970           980           990          1000
        |             |             |             |             |
ACC GAG ACC CTG CTC AAG GGC GCC AAG GAG GTC GAG ACC AAG GAG CAG ATT GCG
Thr Glu Thr Leu Leu Lys Gly Ala Lys Glu Val Glu Thr Lys Glu Gln Ile Ala 144

 1010          1020          1030          1040          1050          1060
  |             |             |             |             |             |
GCC ACC GCA GCG ATT TCG GCG GGT GAC CAG TCC ATC GGT GAC CTG ATC GCC GAG
Ala Thr Ala Ala Ile Ser Ala Gly Asp Gln Ser Ile Gly Asp Leu Ile Ala Glu 162

          1070          1080          1090          1100          1110
           |             |             |             |             |
GCG ATG GAC AAG GTG GGC AAC GAG GGC GTC ATC ACC GTC GAG GAG TCC AAC ACC
Ala Met Asp Lys Val Gly Asn Glu Gly Val Ile Thr Val Glu Glu Ser Asn Thr 180
```

SEQ ID No. 3 (continued)

```
    1120          1130          1140          1150          1160
     |             |             |             |             |
TTT GGG CTG CAG CTC GAG CTC ACC GAG GGT ATG CGG TTC GAC AAG GGC TAC ATC
Phe Gly Leu Gln Leu Glu Leu Thr Glu Gly Met Arg Phe Asp Lys Gly Tyr Ile 198

1170        1180          1190          1200          1210          1220
 |           |             |             |             |             |
TCG GGG TAC TTC GTG ACC GAC CCG GAG CGT CAG GAG GCG GTC CTG GAG GAC CCC
Ser Gly Tyr Phe Val Thr Asp Pro Glu Arg Gln Glu Ala Val Leu Glu Asp Pro 216

       1230          1240          1250          1260          1270
        |             |             |             |             |
TAC ATC CTG CTG GTC AGC TCC AAG GTG TCC ACT GTC AAG GAT CTG CTG CCG CTG
Tyr Ile Leu Leu Val Ser Ser Lys Val Ser Thr Val Lys Asp Leu Leu Pro Leu 234

 1280          1290          1300          1310          1320          1330
  |             |             |             |             |             |
CTC GAG AAG GTC ATC GGA GCC GGT AAG CCG CTG CTG ATC ATC GCC GAG GAC GTC
Leu Glu Lys Val Ile Gly Ala Gly Lys Pro Leu Leu Ile Ile Ala Glu Asp Val 252

          1340          1350          1360          1370          1380
           |             |             |             |             |
GAG GGC GAG GCG CTG TCC ACC CTG GTC GTC AAC AAG ATC CGC GGC ACC TTC AAG
Glu Gly Glu Ala Leu Ser Thr Leu Val Val Asn Lys Ile Arg Gly Thr Phe Lys 270

    1390          1400          1410          1420          1430
     |             |             |             |             |
TCG GTG GCG GTC AAG GCT CCC GGC TTC GGC GAC CGC CGC AAG GCG ATG CTG CAG
Ser Val Ala Val Lys Ala Pro Gly Phe Gly Asp Arg Arg Lys Ala Met Leu Gln 288

1440        1450          1460          1470          1480          1490
 |           |             |             |             |             |
GAT ATG GCC ATT CTC ACC GGT GGT CAG GTG ATC AGC GAA GAG GTC GGC CTG ACG
Asp Met Ala Ile Leu Thr Gly Gly Gln Val Ile Ser Glu Glu Val Gly Leu Thr 306

       1500          1510          1520          1530          1540
        |             |             |             |             |
CTG GAG AAC GCC GAC CTG TCG CTG CTA GGC AAG GCC CGC AAG GTC GTG GTC ACC
Leu Glu Asn Ala Asp Leu Ser Leu Leu Gly Lys Ala Arg Lys Val Val Val Thr 324

 1550          1560          1570          1580          1590          1600
  |             |             |             |             |             |
AAG GAC GAG ACC ACC ATC GTC GAG GGC GCC GGT GAC ACC GAC GCC ATC GCC GGA
Lys Asp Glu Thr Thr Ile Val Glu Gly Ala Gly Asp Thr Asp Ala Ile Ala Gly 342

          1610          1620          1630          1640          1650
           |             |             |             |             |
CGA GTG GCC CAG ATC CGC CAG GAG ATC GAG AAC AGC GAC TCC GAC TAC GAC CGT
Arg Val Ala Gln Ile Arg Gln Glu Ile Glu Asn Ser Asp Ser Asp Tyr Asp Arg 360

    1660          1670          1680          1690          1700
     |             |             |             |             |
GAG AAG CTG CAG GAG CGG CTG GCC AAG CTG GCC GGT GGT GTC GCG GTG ATC AAG
Glu Lys Leu Gln Glu Arg Leu Ala Lys Leu Ala Gly Gly Val Ala Val Ile Lys 378
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2.03.94

SEQ ID No. 3 (continued)

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1710        1720        1730        1740        1750        1760
 |           |           |           |           |           |
GCC GGT GCC GCC ACC GAG GTC GAA CTC AAG GAG CGC AAG CAC CGC ATC GAG GAT
Ala Gly Ala Ala Thr Glu Val Glu Leu Lys Glu Arg Lys His Arg Ile Glu Asp 396

      1770        1780        1790        1800        1810
        |           |           |           |           |
GCG GTT CGC AAT GCC AAG GCC GCC GTC GAG GAG GGC ATC GTC GCC GGT GGG GGT
Ala Val Arg Asn Ala Lys Ala Ala Val Glu Glu Gly Ile Val Ala Gly Gly Gly 414

 1820        1830        1840        1850        1860        1870
   |           |           |           |           |           |
GTG ACG CTG TTG CAA GCG GCC CCG ACC CTG GAC GAG CTG AAG CTC GAA GGC GAC
Val Thr Leu Leu Gln Ala Ala Pro Thr Leu Asp Glu Leu Lys Leu Glu Gly Asp 432

          1880        1890        1900        1910        1920
            |           |           |           |           |
GAG GCG ACC GGC GCC AAC ATC GTG AAG GTG GCG CTG GAG GCC CCG CTG AAG CAG
Glu Ala Thr Gly Ala Asn Ile Val Lys Val Ala Leu Glu Ala Pro Leu Lys Gln 450

   1930        1940        1950        1960        1970
     |           |           |           |           |
ATC GCC TTC AAC TCC GGG CTG GAG CCG GGC GTG GTG GCC GAG AAG GTG CGC AAC
Ile Ala Phe Asn Ser Gly Leu Glu Pro Gly Val Val Ala Glu Lys Val Arg Asn 468

1980        1990        2000        2010        2020        2030
  |           |           |           |           |           |
CTG CCG GCT GGC CAC GGA CTG AAC GCT CAG ACC GGT GTC TAC GAG GAT CTG CTC
Leu Pro Ala Gly His Gly Leu Asn Ala Gln Thr Gly Val Tyr Glu Asp Leu Leu 486

      2040        2050        2060        2070        2080
        |           |           |           |           |
GCT GCC GGC GTT GCT GAC CCG GTC AAG GTG ACC CGT TCG GCG CTG CAG AAT GCG
Ala Ala Gly Val Ala Asp Pro Val Lys Val Thr Arg Ser Ala Leu Gln Asn Ala 504

 2090        2100        2110        2120        2130        2140
   |           |           |           |           |           |
GCG TCC ATC GCG GGG CTG TTC CTG ACC ACC GAG GCC GTC GTT GCC GAC AAG CCG
Ala Ser Ile Ala Gly Leu Phe Leu Thr Thr Glu Ala Val Val Ala Asp Lys Pro 522

          2150        2160        2170        2180        2190
            |           |           |           |           |
GAA AAG GAG AAG GCT TCC GTT CCC GGT GGC GGC GAC ATG GGT GGC ATG GAT TTC
Glu Lys Glu Lys Ala Ser Val Pro Gly Gly Gly Asp Met Gly Gly Met Asp Phe 540

   2200
     |
TGA CCC
 -  Pro
```

3

Pharmaceutical composition for the treatment and prevention of inflammatory diseases and active components of such compositions

The invention pertains to polypeptides containing a part of the aminoacid sequence of the heat shock protein hsp65 of *Mycobacterium tuberculosis* which polypeptides are capable of immunizing against arthritis and other inflammatory diseases and/or curing such diseases, as well as to nucleotide sequences encoding such polypeptides, cells and microorganisms expressing such polypeptides and pharmaceutical and diagnostic compositions containing such polypeptides.

It was found that immunisation of rats with a peptide corresponding to sequence 256-265 of SEQ ID No.1 induced strong protection against induction, seven days later, of adjuvant arthritis (AA). This finding was confirmed when using peptide 256-270. Immunisation with a peptide corresponding to sequence 91-100 of SEQ ID No.1 induced moderate protection, whereas immunisation with peptides corresponding to the other epitopes produces little or no protection against adjuvant arthritis.

The T cell line H.52, originally generated from hsp65 immunised rats and specific for epitope 256-265 also showed a protective effect on AA development when injected i.v. at the time of administration of *Mycobacterium tuberculosis*.

It is concluded that protective epitopes in hsp65 are located at positions where at least 5 aminoacids are in the same relative position as the same aminoacids in a T cell epitope of hsp65 that contains at least 4 consecutive aminoacids which are identical with the corresponding mammalian hsp60 aminoacids. Mammalian hsp includes human, rat and mouse hsp. The human, rat, mouse and mycobacterial hsp60/hsp65 aminoacid sequences are depicted in one letter code in SEQ ID No. 2. The aminoacids which are identical are also shown in SEQ ID No. 2.

The polypeptides are especially those having 5 aminoacids which are in the same relative position as the same aminoacids in one of the sequences 81-100 and 241-270 of SEQ ID No. 1, more particularly having at least 5 aminoacids which are in the same relative position as the same aminoacids in one of the sequences 84-95 and 256-265 of SEQ ID No. 1. Withe preference, the polypeptides comprise at least 7 aminoacids with the same relative positions as those in the hsp65 T cell epitopes. Those epitopes are especially those which have at least 4 consecutive aminoacids which are identical with the corresponding mammalian hsp60 aminoacids. Examples of

suitable polypeptide comprise the sequences [Ala Thr Val Leu Ala], [Ala Leu Ser Thr Leu] and [Leu Ser Thr Leu Val]. In particular, the polypeptide comprises 5-30 aminoacids of the amino acid sequence of hsp65; these hsp65 aminoacids may be coupled to other sequences, such as spacer sequences or fused peptide sequences.

The polypeptides are suitable for protecting against inflammatory diseases, including autoimmune diseases, diabetes, arthritide diseases, atherosclerosis, multiple sclerosis, and myasthenia gravis.

The invention also concerns polypeptide analogues which exhibit the immunological properties of the polypeptides described above, but which contain one or chemical modifications. Such polypeptide analogues, also referred to as peptidomimetics, can e.g. consist of units corresponding to the aminoacid residues of the polypeptides described above, wherein essentially the same side groups are present, but wherein the backbone contains modifications such as substitution of an amide group (CO-NH) by another group such as CH=CH, CO-O, CO-CH_2 or CH_2-CH_2. Other modifications, such as substitutions of an aminoacid by a similar natural, or non-natural aminoacid are also envisaged.

The invention furthermore relates to pharmaceutical compositions suitable for protection against or treatment of an inflammatory disease, including autoimmune diseases, diabetes, arthritide diseases, multiple sclerosis and myasthenia gravis, containing a polypeptide as described above or a nucleotide sequence, an expression system, a cell (eukaryotic) or microorganism corresponding to and/or encoding such polypeptide. The composition may be in the form of a vaccine; it can then also contain a conventional adjuvant, such as Freund's complete or incomplete adjuvant or other adjuvant, and/or carrier materials and other additives.

The composition may also be in the form of a medicine suitable for curing developing or developed inflammatory diseases; it contains conventional additives and excipients. As a treatment composition, it may also contain an antibody against the polypeptides described above.

The invention also relates to diagnostics means and methods based on the polypeptides described above, or the corresponding antibodies or nucleotide sequences (probes).

SEQ. ID No 3. contains the nucleotide sequence and aminoacid sequence of hsp65. Sequences 84-95 and 256-270 correspond to protective polypeptides.

Abbreviations used in this description:

AA, adjuvant arthritis

DDA: dimethyl dioctadecyl ammonium bromide;

hsp60: mammalian 60 kDa heat shock protein

hsp65: mycobacterial 65 kDa heat shock protein

IFA: incomplete Freund's adjuvant

JCA: juvenile chronic arthritis.

Mt: heat-killed *Mycobacterium tuberculosis*

PPD: purified protein derivative of *M.tuberculosis*

PLNC: primed lymph node cells

RA, rheumatoid arthritis

Background

Adjuvant arthritis (AA), an extensively studied model of human rheumatoid arthritis (RA) or reactive arthritis. As the pathogenic mechanisms underlying RA remain unclear, extensive use is made of experimental rodent arthritis models.Lewis rats are susceptible to arthritis following administration of various arthritogenic preparations including heat-killed *M. tuberculosis* (Mt) suspended in IFA (adjuvant arthritis or, AA) (1), streptococcal cell walls (SCW-arthritis), collagen type II and the lipoidal amine CP20961. The cellular basis for AA induction was demonstrated by the passive transfer of the disease to naive rats using splenocytes from arthritic rats (2). Induction of disease in irradiated naive rats by administration of the Mt-reactive T cell clone A2b was reported (3,4). The Ag-specificity of A2b was identified as residues 180-188 of the mycobacterial 65 kDa heat shock protein (hsp65) (5). Attempts to induce AA by immunisation with hsp65 alone proved unsuccessful. Instead, this approach conferred resistance to subsequent attempts to induce AA with whole Mt (5,6). This protective effect is believed to be mediated by T cells specific for hsp65 (7). Preimmunisation with mycobacterial hsp65 has subsequently been reported to confer protection against other forms of experimental arthritis induced with streptococcal cell walls (8), collagen type II (6,9), or synthetic adjuvants such as CP20961 (6) and pristane (10).

Mycobacterial hsp65 belongs to the hsp60 family of heat shock proteins which is highly conserved throughout evolution, and shares 48% aminoacid identity with the mammalian homologue, P1 or hsp60 (11). Expression of mammalian hsp60 is known to be upregulated as a physiological

response to various stressful stimuli, and has been shown to be elevated in inflamed synovia of patients with RA (12),or juvenile chronic arthritis (JCA, ref.13). There have been numerous reports of cross-reactive immunorecognition of mycobacterial hsp65 and endogenous self hsp60 at the T cell level (14-17). This has led to the suggestion that T cells specific for self hsp60 might in some way play a role in the regulation of the inflammatory response (16). Further, this might account for the protective effect of preimmunisation with hsp65 via activation of T cells capable of recognising self hsp60 and leading to improved regulation of future inflammatory episodes induced by administration of arthritogenic substances. Alternatively, immunisation with hsp65 might induce T cell responses to mycobacterial-unique epitopes which are not induced by the Mt administration protocol used to induce AA and these responses might mediate the protective mechanism.

Materials and Methods

Animals: Male inbred Lewis rats ($RT1^l$ MHC haplotype) were obtained from the University of Limburg, Maastricht, The Netherlands. Rats were five to eight weeks old at the start of each experiment.

Antigens and Adjuvants: Heat-killed *Mycobacterium tuberculosis* strain H37Ra (Mt) was obtained from Difco. Purified protein derivative (PPD) of *M.tuberculosis* and purified recombinant hsp65 of *M.bovis* (which is identical to *M.tuberculosis* hsp65) were kindly provided by Dr. J.D.A. van Embden, National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands. Incomplete Freund's adjuvant (IFA, Difco) and dimethyl dioctadecyl ammonium bromide (DDA, Eastman Kodak, Rochester, NY, ref.18) were used as adjuvants. DDA was prepared as a 20 mg/ml suspension in PBS and sonicated to produce a gel which was mixed 1:1 with Ag solution prior to immunisation.

Synthetic peptides: Peptides were prepared by automated simultaneous multiple peptide synthesis (SMPS). The SMPS set-up was developed using a standard autosampler (Gilson 221) as described previously (19). Briefly, for the concurrent synthesis of peptides, standard Fmoc chemistry with Pfp-activated amino acids (Dhbt for serine and threonine) in a sixfold molar excess and Hobt as catalyst were employed. Peptides were obtained as C-terminal amides from 6 mg resin/peptide (0.33 meq/g PAL resin, Millipore).

Two panels of peptides were synthesised, based on the sequences of Mt hsp65 (20) and rat hsp60 (21). Peptides were 15mers with ten amino acid overlap with each adjacent peptide (i.e residues 1-15, 6-20, 11-25 etc). Thus, every possible 11mer sequence of each protein was contained within a peptide.

Immunisations and primed lymph node populations: Rats were immunised with either Mt or hsp65. Mt was suspended at 5 mg/ml in IFA or DDA and 100 µl injected in each hind footpad (i.e. 500µg/footpad, 1 mg/rat). HSP65 (1mg/ml in PBS) was mixed 1:1 with DDA and 100 µl injected (i.e. 50 µg/footpad, 100 µg/rat). Ten to 21 days later, draining popliteal lymph nodes were removed, disaggregated, washed three times and used as a source of primed lymph node cells (PLNC). In control experiments splenocytes and lymph nodes from unimmunised rats and PLNC from rats immunised with IFA or DDA/PBS alone were used.

Immunizations with synthetic peptides and primed lymph node populations: Rats were immunized with 50µg of synthetic peptide in PBS/DDA in each hind footpad (i.e. 50 µg/footpad, 100 µg/rat). Ten days later, draining popliteal lymph nodes were removed, disaggregated, washed three times and used as a source of primed lymph node cells (PLNC). In some experiments, PLNC were derived as pooled inguinal and popliteal lymph nodes from AA rats 35-42 days post Mt immunization.

Tissue culture reagents: Iscove's modification Dulbecco's medium (IMDM, Gibco) supplemented with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Gibco) and 5×10^{-5} M 2-ME was used as culture medium. Cell populations were washed in IMDM without supplements.

T cell proliferation assays: PLNC were cultured in triplicate in 200 µl flat bottom microtitre wells (Costar) at 2×10^5 cells per well with or without antigen. In initial experiments PLNC were tested for responsiveness to individual peptides at concentrations of 5 and 20 µg/ml, and Mt, hsp65 and PPD over a range of concentrations. Concanavalin A (2 µg/ml) was used as a positive control for T cell proliferation. Cultures were incubated for 96 h at 37°C in a humidified atmosphere of 5% CO_2. Cultures were pulsed for the final 16 h with [^{3}H] TdR (Amersham, U.K., 1 µCi/well) and TdR uptake measured using a liquid scintillation β counter.

Assays using T cell lines were performed as above using $2x10^4$ line cells per well with irradiated (30 Gy) syngeneic accessory cells (either $3x10^5$ splenocytes/well or 10^6 thymocytes/well).
Results are expressed as mean counts per minute (cpm) of triplicate cultures. In experiments where responses to Ag were low, responses were considered significant if the stimulation index (S.I. = mean cpm with Ag - mean cpm without Ag) was greater than two and Student's t tests gave $p < 0.01$.

T cell lines: T cell lines with specificity for mycobacterial hsp65 or peptides were generated by bulk stimulation of either hsp65-PLNC or Mt-PLNC. PLNC were cultured at $5x10^6$/ml in culture medium supplemented with 2% normal rat serum (NRS) in the presence of 10 µg/ml Ag. After three days viable cells were harvested using a ficoll-isopaque gradient and cultured for a further four days in culture medium + 5% FCS and 5% TCGF (Con A-activated rat spleen supernatant). Seven days after initial stimulation lines were restimulated with irradiated spleen accessory cells and 10 µg/ml Ag in culture medium + NRS. Lines were maintained in this seven day restimulation cycle.

Short term epitope-specific T cell lines were also generated from rats immunized with synthetic peptides. Peptide-PLNC were cultured as above in the presence of 10 µg/ml peptide.

Monoclonal antibodies: Anti-MHC monoclonal antibodies were added to proliferation assays to determine the MHC-restriction of responses to hsp65 and peptides. OX6 (anti-RT1.B, class II), OX17 (anti-RT1.D, class II), OX18 (anti-RT1.A, class I) and UD15 (anti-chloramphenicol control antibody) were used. All antibodies were mouse IgG1.

FACS Analysis: FACS analysis was used to phenotype T cell lines. Cells were incubated with either R73 (anti-αβ TCR), W3/25 (anti-CD4) or OX8 (anti-CD8), all mouse IgG1 antibodies. Second step staining was with FITC-conjugated Goat anti-Mouse Ig (Becton Dickinson). Cells were analysed using a Becton Dickinson FACScan analyser.

Induction and clinical assessment of xperimental arthritis: Arthritis was induced by a single intradermal injection in the base of the tail. AA was

induced with 0.5mg Mt suspended in 100µl IFA. CP20961 arthritis was induced with 2 mg CP20961 in 100 µl light mineral oil (Sigma). Rats were examined daily for clinical signs of arthritis. Severity of arthritis was assessed by scoring each paw from zero to four based on degree of swelling, erythema and deformity of the joints. Thus the maximum possible arthritis score was 16.

Modulation of arthritis with hsp65 peptides and epitope-specific T cell lines: Synthetic peptides corresponding to eight T cell epitopes present in mycobacterial hsp65 were tested for protective effects on arthritis development. Rats were immunized with 100 µg of individual peptide seven days prior to arthritis induction. Peptides were immunized in each hind footpad in 10 mg/ml DDA (50 µl/pad). Control rats received only PBS/DDA. Epitope-specific T cell lines were also tested for protective activity by intravenous administration of lines at the time of arthritis induction. T cell lines were restimulated *in vitro* with irradiated spleen APC and specific peptide. Four days later T cell blasts were harvested by ficoll gradient, washed and applied to a second ficoll gradient to remove any contaminating APC. T cells were washed twice in wash medium and twice in PBS and finally suspended at $2.5x10^7$/ml in PBS. Immediately prior to injection of Mt, 200 µl (i.e. $5x10^6$) T cells were injected i.v. in the tail vein.

Results

Primed lymph node cell responses to Mt, PPD and hsp65.

Analysis of proliferative responses revealed that naive splenocytes or PLNC from rats immunised with IFA or DDA/PBS responded to some extent to whole Mt without prior immunisation (Fig. 1). These responses were not due to the presence of hsp65 which did not stimulate these populations.

Immunisation with Mt/IFA produced increased responsiveness to Mt and also induced strong responses to PPD and low level responsiveness to hsp65, indicating that Mt immunisation could prime hsp65-specific T cells.

Immunisation with hsp65 primed for responsiveness to hsp65. PPD (which contains native hsp65) also induced proliferation indicating that immunisation with recombinant hsp65 activated T cells recognising native mycobacterial hsp65. Hsp65-PLNC showed increased responsiveness to Mt compared to DDA/PBS-PLNC. Hsp65-PLNC proliferative responses to hsp65 were effectively inhibited by addition of the OX6 monoclonal antibody to

cultures, indicating that the response was predominantly (if not totally) restricted to the RT1.B^{l} MHC class II molecule (Fig. 2). No significant inhibition was seen using anti-RT1.D, anti-RT1.A or anti-chloramphenicol isotype control mAbs.

Identification of hsp65 T cell epitopes following immunisation with hsp65

Hsp65-PLNC were analysed for responsiveness to a panel of peptides covering the entire sequence of hsp65 (Fig. 3a). Several peptides induced significant proliferation, suggesting the presence of seven epitopes. In terms of magnitude of response, three regions of the molecule (residues 176-190, 211-230 and 221-240) appeared to contain the "dominant" T cell epitopes, while regions 86-100, 251-270, 396-410 and 506-525 contained "subdominant" or minor epitopes. For four of these epitopes (211-230, 221-240, 251-270 and 506-525) responses to adjacent overlapping peptides were seen, suggesting that the core epitopes lay within 216-225, 226-235, 256-265 and 511-520 respectively.

Peptide 176-190 contained the AA-associated epitope 180-188. A synthetic peptide 180-188 was also tested. It was found to induce responses, but at a lower level than the longer 176-190 peptide, indicating that the minimal epitope provides less efficient stimulation at the PLNC level.

PLNC responses were tested at ten, 14 and 21 days post immunisation. Responses were strongest at day ten and declined with time. The pattern of dominance remained constant and no "new" responses to different peptides were observed at day 21. Initial experiments used pooled PLNC from several immunised rats. Experiments testing the responsiveness of individual rats showed no variation between rats in the pattern of responsiveness. Peptides that stimulated hsp65-PLNC were also tested on unprimed splenocytes and DDA/PBS-PLNC and were found to have no stimulatory activity, indicating that immunisation with hsp65 was required to prime for the peptide induced responses.

Hsp65-PLNC were also tested for responses to a panel of peptides covering the entire sequence of rat hsp60. None of the rat hsp60 peptides induced significant responses in hsp65-PLNC tested at ten or 21 days post immunisation.

To confirm the presence of the hsp65 T cell epitopes identified at the PLNC level, short term T cell lines were established from ten day hsp65-PLNC by bulk stimulation with hsp65. Seven days after the first restimulation, these lines were tested against the entire panel of hsp65

peptides (Fig. 3b). Surprisingly the response pattern differed from that of the original PLNC populations. The three dominant epitopes, 176-190, 216-225 and 226-235, all induced strong responses. Epitopes 256-265, 396-410 and 511-520 were also recognised, but responses to 86-105 were absent. Conversely, two peptides that failed to stimulate hsp65-PLNC (386-400 and 446-460) did stimulate the hsp65-specific lines. This alteration in response pattern following a single *in vitro* restimulation was observed in four separate experiments. None of the hsp65-specific lines showed significant responses to any rat hsp60 peptides.

T cell responses to hsp65 epitopes following immunisation with whole *M.tuberculosis*.

Mt-PLNC were tested for responses to hsp65 peptides, to determine whether immunisation with whole Mt could prime for responsiveness to the hsp65 epitopes described above. Immunisation with Mt/IFA (i.e. the AA-inducing protocol) consistently failed to induce significant responses to hsp65 peptides (data not shown). As Mt/IFA immunisation induced only low level reactivity to hsp65 (Fig. 1), we tested PLNC from rats immunised with Mt mixed with DDA. This protocol, (using 500 µg or 1 mg of Mt per rat) produced PLNC showing increased reactivity to hsp65, and significant responses to hsp65 peptides (Figs. 1 and 4a). The response pattern differed from that obtained using hsp65-PLNC. Epitope 176-190 appeared to be dominant, epitopes 216-225, 226-235, 256-265 and 511-520 were minor and responses to epitopes 86-105 and 396-410 absent (Fig. 4a).

Hsp65-restimulated T cell lines established from Mt/IFA-PLNC showed clear responses to hsp65 peptides (Fig. 4b), indicating that although responses to peptides could not be detected at the PLNC level, Mt/IFA immunisation did prime hsp65-specific T cells. After one *in vitro* stimulation with hsp65, epitope 176-190 was dominant, with epitopes 216-225, 226-240, 256-265 and 511-520 minor. No response could be detected to epitopes 86-100 or 396-410, suggesting that T cells specific for these epitopes are activated *in vivo* following immunisation with hsp65 but not whole Mt. Lines restimulated with Mt showed an even stronger dominance of the response to epitope 176-190. In contrast to hsp65-restimulation, responses to epitope 256-265 were absent. Responses to peptides 386-400 and 446-460 were detected in lines from Mt immunised rats after restimulation with hsp65 but not Mt. Lines from Mt/DDA-PLNC showed identical response patterns.

Analysis of T cell lines specific for defined hsp65 epitopes

T cell lines were generated by restimulation of hsp65-PLNC with individual synthetic peptides. For epitopes where responses had been detected to two overlapping peptides, the line was generated using the peptide that induced strongest proliferation of hsp65-PLNC (e.g. for epitope 216-225, peptide 211-225 was used). This resulted in eight T cell lines. Table I summarises the response patterns to each epitope and names each peptide-specific T cell line. No lines specific for peptide 386-400 were established.

After at least four *in vitro* restimulations each line was tested for responsiveness to specific peptide, hsp65, Mt and PPD. To test for cross-reactivity with self hsp60, each line was also tested against the corresponding rat hsp60 peptide (Fig. 5).

All lines responded to hsp65 and their specific peptides and, in all but one instance, an overlapping peptide. Between residues 211 and 235 four overlapping peptides were stimulatory. Thus it was possible that four separate epitopes were present in this region. However, T cell lines generated against peptides 216-230 and 221-235 responded more strongly to peptides 211-225 and 226-240 respectively, indicating the presence of only two epitopes: 216-225 and 226-235 as described above. Line H.36, specific for peptide 176-190 also responded to a peptide including residues 180-188, previously described to be recognised by the arthritogenic T cell clone A2b, indicating that these two lines recognise the same core epitope.

While all lines responded to Mt to some extent, the level of proliferation varied between lines. Lines H.36 and H.46 both showed strong responses to Mt, while the other six lines responded poorly. All lines responded to PPD.

Line H.52, specific for residues 256-265, showed clear responses to the homologous peptide of rat hsp60. Therefore, while no responses to rat hsp60 peptides could be detected using hsp65-PLNC or the hsp65-specific lines, cross-reactive T cell recognition could be demonstrated using this epitope-specific cell line. The mycobacterial peptide induced a five to ten fold higher level of proliferation than the rat peptide. This might explain why the rat peptide was not recognised at the PLNC level as responses to the mycobacterial peptide were minor and any decrease in stimulatory activity could result in responses below the detectable level. The core epitope recognised by H.52 contains only three residues which differ from the rat hsp60 sequence, situated at the C terminal end of the epitope (see

Table I). All other T cell lines failed to respond to rat hsp60 peptides. Thus, of the nine T cell epitopes in mycobacterial hsp65 identified by this study, only one showed cross-reactivity with rat hsp60.

All peptide-specific or hsp65-specific T cell lines were analysed for T cell phenotype and MHC-restriction. FACS analysis showed that all T cell lines were αβ TCR^+ $CD4^+$ $CD8^-$. MHC-restriction was determined by assessing the ability of anti-MHC monoclonal antibodies (10 µg/ml) to inhibit hsp65 or peptide (1 µg/ml) induced responses in proliferation assays. Addition of anti-RT1.B reduced proliferation of all lines by greater than 70%, while anti-RT1.D or anti-RT1.A had no significant effect (Fig. 6). Thus all lines were RT1 B^l-restricted.

Immunization with hsp65 peptides primes for epitope-specific T cell responses

To determine whether immunization of rats with synthetic peptides was effective at priming for T cell reactivity to hsp65 epitopes, we examined *in vitro* proliferative responses following immunization with 100µg of peptide each containing individual T cell epitopes. PLNC isolated ten days after immunization were tested for responsiveness to the immunizing peptide, overlapping peptides and to hsp65 (Table II). PLNC responses were observed after immunization with seven of the nine peptides. Responses were not seen after immunization with peptides 386-400 and 511-525.

Bulk stimulation generated T cell lines to eight of the nine peptides tested. These eight lines were tested for specificity after four *in vitro* restimulations (Table II). All responded to the immunizing peptide and, to some extent, to hsp65. Also, the lines were tested for responsiveness to overlapping peptides and, in all but one case, showed identical response patterns to T cell lines generated previously against the same epitopes after immunization with whole hsp65. These findings suggest that T cells activated *in vivo* by immunization with hsp65 or synthetic peptides recognize the same core epitopes.

A T cell line specific for th 86-100 peptide generated from rats immunized with hsp65 responded to peptides 86-100 and 91-105 (i.e. recogned a core epitope of 91-100). The T cell line generated following immunization with the 86-100 peptide also responded to peptides 81-95 and 91-105. This suggested the presence of two T cell populations, one recognising residues 86-95, the other recognising residues 91-100. Interestingly, the sequences of mycobacterial hsp65 and rat hsp60 covering residues 86-95 are identical.

Accordingly, the line proliferated in response to peptides corresponding to rat hsp60 81-95 and 86-100. Thus immunization with the hsp65 86-100 peptides could prime for responsiveness to an epitope in self hsp60.

Line H.52 (generated from hsp65-immunized rats and recognizing epitope 256-265) also responded to the highly homologous rat hsp60 256-270 peptide (see above). Accordingly the 256-270 specific T cell line derived from peptide immunized rats also responded to rat 256-270 (Table II). This line also showed an increased "autoreactive" response to syngeneic APC which had been heat-shocked (one hour at 42°C prior to irradiation) in comparison control APC cultured at 37°C (Table III). This suggested that increased expression of endogenous hsp60 by APC results in presentation of this cross-reactive epitope in association with MHC for T cell recognition.

Analysis of the ability of hsp65 peptides to vaccinate against arthritis

The effects of preimmunization with synthetic peptides containing individual epitopes on the development of AA were analysed (Fig. 7). The eight peptides which primed for epitope specific T cells were tested. Rats were immunized with 100µg of peptide seven days prior to AA-induction with Mt.

Preimmunization with peptide 256-270 resulted in clear protective effects against AA development. The mean maximum arthritis score after 256-270 preimmunization was 1.7 (24 rats in five separate experiments) compared to mean maximum score of 11.5 for control rats preimmunized with PBS. Of the 24 rats preimmunized with 256-270, twelve did develop clinical signs of arthritis, which were milder than those developed by control rats. Also, whereas control rats suffered permanent joint deformities that persisted after the initial arthritis had subsided, the 256-270 preimmunized rats that developed AA were remarkably free of permanent deformities. Preimmunization with the other peptides containing hsp65 T cell epitopes had no significant effects on the onset of AA.

PLNC from preimmunized rats were tested for responsiveness to hsp65 epitopes 42 days after Mt administration (Fig. 8). PLNC from rats preimmunized with PBS/DDA alone showed significant responses to peptide 176-190 (containing the AA-associated 180-188 epitope) but not to any other of the defined hsp65 epitopes. PLNC from rats preimmunized with individual hsp65 peptides all showed responses to the immunizing peptide, although the level of proliferation varied. Thus, whereas preimmunization with peptides 211-225 and 446-460 induced strong responses, preimmunization with peptide

256-270 (which protected against AA) induced marginal PLNC responses. Responses to peptide 176-190 containing the AA-associated epitope were found in all PLNC populations (and enhanced after 176-190 preimmunization) with one exception. PLNC from rats protected from AA by preimmunization with peptide 256-270 showed a total lack of response to 176-190.

Administration of a T cell line specific for the cross-reactive 256-265 epitope confers protection against AA

Preimmunization with hsp65(256-270) protected against arthritis. To determine whether this activity was the result of activation of T cells specific for this epitope, the effects of epitope-specific T cell lines on AA were tested (Fig. 9). T cell lines were administered to rats at the same time as Mt for AA induction ($5x10^6$ T cells per rat injected i.v. in a tail vein). In two experiments, administration of line H.52 recognising the 256-265 epitope clearly reduced the severity of AA compared to control animals receiving PBS. The T cell lines H.36, H.43 and H.46 (specific for epitopes 180-188, 216-225 and 226-235 respectively) were also tested and had no significant effects on AA development.

Line H.46 was restimulated with specific peptide (226-240) in the presence of 10 µg/ml peptide 256-270, in order to ensure that the protective effect of line H.52 was not the result of administration of residual 256-270 peptide carried over from the *in vitro* restimulation of the line. Administration of the resulting 226-235 specific T cell blasts failed to protect against AA.

Preimmunization with peptide 256-270 also vaccinates against CP20961-induced arthritis

The only hsp65 peptide which protected strongly against AA was found to be 256-270, which induced T cell reactivity against the corresponding sequence of rat hsp60. This finding suggested that self-reactive T cells account for hsp65-induced protection against arthritis. This mechanism would then be expected to protect against arthritis induced by other compounds and not be dependent on the use of mycobacteria. Therefore the capacity of peptide 256-270 to induce protection in the CP20961-induced model was tested. As CP20961 is a lipoidal amine, there is no possibility of this arthritogenic preparation containing an antigenic component with potential cross-reactivity with hsp65 256-270. Preimmunization with hsp65 256-270 strongly protected rats against CP20961-induced arthritis, whereas

a control peptide (hsp65 211-225) did not (Fig. 10). Thus preimmunization with hsp65 256-270 can protect against AA and CP20961-induced arthritis, presumably via a mechanism which is not dependent on the administration of mycobacteria as a component of the arthritogenic inoculation.

Rat hsp60(256-270) fails to vaccinate against arthritis

Since preimmunization with hsp65 256-270 provided protection against AA and T cells specific for this peptide also responded to the highly homologous peptide based on the rat hsp60 256-270 sequence, the rat peptide was tested for similar protective effects (Fig. 11). Protection was observed after preimmunization with mycobacterial hsp65 256-270, but not with rat hsp60 256-270. An explanation for this discrepancy may be that immunization with rat hsp60 256-270 does not prime for T cell responses. To investigate this we generated peptide-specific T cell line from rat 256-270 (R.256-270) immunized PLNC and compared responses of this line with that of a line from mycobacterial 256-270 (M.256-270) immunized PLNC (Fig. 12). The line generated against M.256-270 responded to peptides representing M.256-270, M.251-265 and a shorter "core" peptide M.256-265, and cross-reacted with R.256-270 and (weakly) R.256-265. In contrast, the line generated against R.256-270 responded to R.256-270, but not to M.256-270 or to R.256-265 and M.256-265. This line also showed a high "autoreactive" response to APC alone, suggesting that the APC were already expressing the self hsp60 epitope.

Thus immunization with M.256-270 primed for T cell responses that cross-reacted with R.256-270, but immunization with R.256-270 primed for rat-specific T cell responses that did not cross-react with M.256-270. The fact that the R.256-270 specific line did not respond to R.251-265 or R.256-265 suggests that one or more of the five C-terminal residues of R.256-270 is important for stimulation of this line. None of these five residues share identity with the mycobacterial sequence, presumably accounting for the lack of responsiveness to M.256-270.

Furthermore, using anti-MHC mAbs, the T cell lines generated against M.256-270 and R.256-270 were found to have different MHC-restrictions. Peptide induced proliferation of the M.256-270 specific line was inhibited by the OX6 anti-RT1.B mAb, whereas the R.256-270 specific line was inhibited by the OX17 anti-RT1.D mAb.

Table I

Mycobacterial hsp65 epitopes recognised by Lewis rat T cells.

Immunisation		hsp	hsp	Mt	Mt	Mt	hsp	
In vitro stimulation		-	hsp	-	hsp	Mt	peptide	
Epitope	Sequence[1]							T cell line
91-100	DGTTTATVLAQALVR	+	-	-	-	-	+[2]	H.18
176-190	EESNTFGLQLELTEG	+++	+++	+++	+++	+++	+	H.36
216-225	AVLEDPYILLVSSKV	+++	+++	+	+	+	+	H.43
226-235	STVKDLLPLLEKVIG	+++	+++	+	+	+	+	H.46
256-265	ALSTLVVNKIRGTFK	+	+	+	+	-	+	H.52
386-400	ELKERKHRIEDAVRN	-	+	-	+	-	-	-
396-405	DAVRNAKAAVEEGIV	+	+	-	-	-	+	H.80
446-455	APLKQIAFNSGLEPG	-	+	-	+	-	+	H.90
511-520	FLTTEAVVADKPEKE	+	+	+	+	+	+	H.103

Footnotes

1. The sequences of hsp65 peptides used to generate each line are shown. Core epitopes, as defined by responses to overlapping peptides, are denoted by underlined residues. Residues sharing identity with the corresponding sequence of rat hsp60 are in bold.
Differential recognition of epitopes following differing immunisation and restimulation protocols are summarised.
- No response
+ Minor response
+++ Dominant response
2. + and - in this column refer to whether a peptide-specific T cell line was generated from hsp65-PLNC.

Table II

Immunization with hsp65 peptides primes for T cell responses.
Rats were immunized with synthetic peptides containing individual hsp65 epitopes (100µg peptide/DDA per rat). Ten days later PLNC were isolated and tested for responses to overlapping peptides (20 µg/ml). Peptide-specific T cell lines were generated by bulk *in vitro* stimulation of PLNC with immunizing peptide. Lines were tested for responses to overlapping peptides (10 µg/ml). All PLNC and T cell lines showed signficant responses to 20 µg/ml hsp65 (data not shown). Results are expressed as mean cpm of triplicate cultures. All SEM were less than 20%.

Table II

Immunizing peptide		*In vitro* peptide	CPM PLNC	CPM T Cell Line	Response after hsp65 immunization[1]
86-100		0	2518	1127	
		81-95	15296	15785	-
		86-100	57925	49422	+
		91-105	1999	18444	+
	Rat	86-100	6196	25535	-
176-190		0	1784	1255	
		171-185	3082	1640	-
		176-190	57707	31400	+
		181-195	1916	1653	-
		180-188	7859	23275	+
211-225		0	986	1479	
		206-220	645	988	-
		211-225	39765	121978	+
		216-230	11117	102341	+
226-240		0	1286	882	
		221-235	7381	100306	+
		226-240	22761	152071	+
		231-245	1655	862	-
256-270		0	2448	762	
		251-265	6391	66063	+
		256-270	16423	69037	+
		261-275	2553	646	-
	Rat	256-270	4152	14987	+
396-410		0	3399	2095	
		391-405	2280	39335	+
		396-410	8477	88916	+
		401-415	3211	2011	-
446-460		0	1928	1028	
		441-455	4715	17805	+
		446-460	22918	38324	+
		451-465	2130	1283	-
511-525		0	2804	537	
		506-520	2754	61293	+
		511-525	3164	129373	+
		516-530	2821	1231	-

[1]. As determined using T cell lines generated from hsp65-immunized rats.

Table III

Heat-shocked APC stimulate hsp65(256-270) specific T cells.
T cell lines ($2x10^4$/well) were cultured with APC ($2x10^5$/well) that had been cultured for one hour at either 37°C or 42°C prior to irradiation. Cells were cultured with or without specific peptide as Ag (10µg/ml). Line H.43, specific for the non-cross-reactive, mycobacterial hsp65 unique, epitope 211-225 was used as a control. Results are expressed as mean cpm of triplicate cultures. All SEM were less than 20%.

T cell line	P.m52.1	Line H.46
Specificity	256-265	226-235
T cells - APC	44	22
37°C APC - Ag	470	33
+ Myco. pept	120744	162785
+ rat pept	18061	NT
42°C APC - Ag	15960	37
+ Myco. pept	115626	150887
+ rat pept	25842	NT

Discussion

Immunisation of Lewis rats with heat-killed Mt in IFA induces AA (1), reported to be associated with T cell responses to residues 180-188 of mycobacterial hsp65 (5). Conversely, immunisation with hsp65 protects against subsequent attempts to induce AA by an as yet undefined T cell-mediated mechanism (5,6). Epitopes within hsp65 recognised by Lewis rat T cells were identified in this description. Responsiveness to these epitopes in T cell populations following immunisation with either Mt or hsp65 was compared.

Immunisation of rats with recombinant mycobacterial hsp65 primed for MHC class II (RT1.B^l)-restricted recognition of hsp65. Analysis of hsp65-PLNC responses to overlapping peptides covering the entire hsp65 sequence identified seven T cell epitopes. Following a single *in vitro* restimulation with hsp65, two further epitopes were identified. Thus Lewis rat T cells recognise nine epitopes in mycobacterial hsp65.

Immunisation with Mt suspended in IFA (as used in the AA-inducing protocol) did not prime for PLNC responses to hsp65 peptides. However, Mt/DDA immunisation did induce significant responses to hsp65 peptides.

Significantly, AA only develops following Mt/IFA immunisation and not after Mt/DDA immunisation (S.M.A, unpublished observations). Thus the enhanced activation of hsp65-specific T cells when DDA is used as adjuvant might account for this difference. T cells from Mt/IFA and Mt/DDA immunised rats showed identical response patterns after *in vitro* restimulation with hsp65 indicating that Mt/IFA does prime for T cell recognition of hsp65, but at a relatively low level.

Patterns of dominance of hsp65 epitopes differed following immunisation with hsp65 or whole Mt. Hsp65 immunisation resulted in three co-dominant epitopes: 176-190, 216-225 and 226-235. After Mt immunisation, epitope 176-190 appeared dominant with epitopes 216-225 and 226-235 being minor along with 256-265 and 511-520. The dominance of the 176-190 epitope was even more marked when Mt-PLNC were restimulated with Mt. Hsp65, but not Mt immunisation primed for responses to epitopes 86-100 and 396-410.

We generated αβ TCR^+CD4^+, $RT1.B^1$-restricted T cell lines specific for eight of the nine epitopes identified. Of these eight T cell lines two, H.36 and H.46 (which recognise 176-190 and 226-235 respectively) responded strongly to Mt while the others responded relatively weakly. This is consistent with the dominance of 176-190 following immunisation with Mt.

The relatively low quantities of hsp65 present in the Mt preparation might result in focussing of T cell responses on hsp65 epitopes with higher affinities for MHC class II molecules. Preliminary experiments testing the ability of peptides containing hsp65 epitopes to inhibit proliferation of T cell lines with other Ag specificities suggest that peptide 176-190 may have a higher affinity for $RT1.B^1$ molecules than do peptides 211-225 or 226-240 (data not shown). Thus, when Ag concentrations are limited (i.e. following Mt immunisation) dominance of T cell epitopes might be more dependent on their relative MHC binding affinities, whereas following immunisation with large quantities of specific antigen (50-100 µg hsp65 per rat) relative dominance might not be simply a function of affinity for MHC. Clearly, antigen processing of the intact hsp65 molecule will determine the peptide fragments generated during *in vivo* priming, and the naturally processed fragments will not be identical to the synthetic 15mers used in this study. Also the molecular context of hsp65 (i.e recombinant monomeric hsp65, or the multimeric native protein in the Mt preparation) could affect antigen processing and influence the pattern of epitope recognition.

Significantly, the dominant hsp65 pitope following Mt immunisation, 176-190, contains the 180-188 sequence previously reported to be recognised

by the arthritogenic T cell clone A2b. Therefore, the AA-inducing protocol results in a T cell response skewed towards the AA-associated epitope. For PLNC responses, the longer 176-190 peptide induced stronger proliferative responses than the minimal 180-188 peptide. This is a significant finding as previous studies have analysed polyclonal responses to 180-188 (7,22). The length of naturally processed peptides found in the binding groove of MHC class II molecules has been described as 13 to 25 amino acids (23,24). Thus a more stable interaction of the 15mer 176-190 with the RT1.B^1 molecule compared with the 9mer 180-188 might account for the increased stimulatory activity.

Of the nine hsp65 T cell epitopes defined by this study, one was cross-reactive with rat hsp60. Although no significant response to any rat hsp60 peptide was observed at the PLNC level following immunisation with either hsp65 or Mt, the T cell line H.52, specific for the 256-265 epitope did respond to the corresponding rat hsp60 peptide. Accordingly this region is highly conserved (mycobacterial: ALSTLVVNKI, rat: ALSTLVLNRL) with seven residues identical and conservative substitutions at the other three positions. The region 243-265 shows highest identity between mycobacterial and mammalian hsp60s, with 18/23 residues identical and five conservative substitutions (11).

The present findings provide important insights into T cell mediated protective effect of hsp65 preimmunisation in experimental models of arthritis, for which three possible mechanisms have been proposed. Firstly, previous data suggested that hsp65 preimmunisation might down-regulate the response to the AA-associated 180-188 epitope (22). The results of the present study do not support this, as 176-190 is a co-dominant epitope following hsp65 immunisation.

Secondly, enhanced activation of T cells (recognising one or more hsp65 epitope) following preimmunisation might result in a more efficient recognition and clearance of Mt on subsequent challenge, before AA can develop. It was found that hsp65-immunisation results in improved recognition of hsp65 epitopes compared to Mt-immunisation (both in terms of number of epitopes recognised and magnitude of responses). These differences might form the basis for such a protective mechanism.

Reports of T cell recognition of epitopes conserved between mycobacterial hsp65 and mammalian hsp60 (14-17) have led to a third hypothesis, in which preimmunisation with hsp65 activates T cells recognising cross-reactive epitopes (16). Subsequent recognition of self hsp60 upregulated

during inflammation within the joint would then regulate the inflammatory process, preventing development of chronic arthritis. If this hypothesis is correct, the relevant T cell epitope (for models using Lewis rats) must be residues 256-265 as this is the only cross-reactive epitope recognised following hsp65 immunisation. Interestingly, epitope 256-265 was recognised only poorly by Mt/IFA immunisation. Also, line H.52 responded weakly to Mt. Therefore the AA-inducing protocol is poor at activating T cells specific for this epitope.

Preimmunisation with hsp65 not only protects against AA, but also against arthritis induced without mycobacteria (6-10) and, in pristane (10) and CP20961-induced (6) arthritis, without any exogenously added protein. Therefore it is probable that the pathogenic mechanisms in these models differ, yet all can be prevented by preimmunisation with hsp65. With this in mind, the hypothesis which does not require recognition of whole Mt, but involves cross-reactive T cell recognition of mycobacterial hsp65 and rat hsp60 seems most attractive as protection could be accounted for by a single mechanism, regardless of the arthritogenic agent used. T cell recognition of self hsp60 might also be relevant in resistance to human arthritic conditions. T cell reactivity to self hsp60 has been reported in patients with RA (25) and JCA (26). An exciting extension of this was the report of two $CD4^+$ T cell clones from a JCA patient which recognised cross-reactive epitopes in the highly conserved region (243-265) recognised by the H.52 T cell line in our study, and that the donor patient had a favourable outcome of disease (17). Also epitopes in this region of self hsp60 were recognised by $CD4^+$ CTL from healthy human donors (14).

T cell reactivity against hsp65 is believed to be involved in the protective mechanism(s). Rats were preimmunized with synthetic peptides containing individual epitopes seven days prior to AA induction with Mt. This approach led to a striking protective effect in rats preimmunized with peptide 256-270. None of the other peptides tested showed any influence on AA development. The T cell line H.52, originally generated from hsp65 immunized rats and specific for epitope 256-265, also showed a protective effect on AA development when injected i.v. at Mt administration. Transfer of line H.52 did not induce total protection against AA but clearly reduced the severity of the arthritis. The low numbers of cells transferred in this study ($5x10^6$ compared with $5x10^7$-10^8 in other studies), enforced due to the slow rate of growth displayed by H.52, might be insufficient to induce a fully protective effect.

The finding that preimmunization with peptide 176-190 (containing the AA-associated 180-188 epitope) had no effect on AA onset is in contrast with previous reports that preimmunization with peptides 180-188 induces effective protection against AA. A possible explanation for this difference lies in the different preimmunization regimens adopted. We immunized with peptide in the footpads seven days prior to Mt immunization using DDA as adjuvant, whereas the previous studies immunized i.p. with peptide in IFA on days -35, -20 and -5. Although i.p. immunization with peptide in IFA has been used to induce epitope specific T cell tolerance, this approach was reported to induce 180-188 specific T cells capable of transferring protection to naive rats. These findings could not be repeated.

No protective effect was found of administering the 180-188-specific T cell line H.36 at the time of Mt immunization. This contrasts with the previously described protective effects of transferring spleen T cells from 180-188 immunized rats or the 180-188 specific T cell lines A2 and A2c. H.36 might therefore be an "A2b-like" line with AA inducing rather than protective activity, although transfer of H.36 did not increase severity of AA and we have not tested the ability of the line to induce AA in irradiated rats. Alternatively, the low numbers of cells transferred in this study again might not be sufficient to induce a protective effect.

Line H.52 has been shown to recognize the 256-270 sequence of rat hsp60. Similarly, short term T cell lines derived from rats immunized with peptide 256-270 (generated both from rats ten days after peptide immunization and from protected rats 42 days after Mt administration) responded to the corresponding rat hsp60 peptide. These lines also showed small but significant responses to heat shocked APC indicating that the endogenous self hsp60 epitope could be presented in association with MHC class II for T cell recognition when hsp60 levels were upregulated.

Thus, immunization with mycobacterial hsp65 256-270 protected against AA development and activated T cells capable of responding to the shared epitope in rat hsp60. This finding provides support for the hypothesis that the mechanism by which hsp65 preimmunization protects Lewis rats against arthritis is based on activation of T cells that recognize an epitope shared with rat hsp60. Recognition by these T cells of elevated levels of the self epitope presented by MHC class II expressing cells at the site of inflammation (the joint) would then provide an antigen-specific mechanism for regulation of the inflammatory process. This mechanism does not require the "protective" T cell to recognize a mycobacterial component

and therefore provides an attractive single mechanism for explaining hsp65-induced resistance to models not employing bacterial derived-arthritogens. The most notable of these models in the Lewis rat is the model induced with CP20961, which is a lipoidal amine and therefore has no possible antigenic cross-reactivity with hsp65. It was found indeed that peptide 256-270 confers protection against the CP20961-induced model.

References

1. Pearson, C. M. 1956. Development of arthritis, periarthritis and periostitis in rats given adjuvant. *Proc. Soc. Exp. Biol. Med. 91:101.*

2. Pearson, C. M. and F. D. Wood. 1964. Passive transfer of adjuvant arthritis by lymph node or spleen cells. *J. Exp. Med. 120:547.*

3. Holoshitz, J., Y. Naparstek, A. Ben-Nun and I. R. Cohen. 1983. Lines of T lymphocytes induce or vaccinate against autoimmune arthritis. *Science. 219:56.*

4. Van Eden, W., J. Holoshitz, Z. Nevo, A. Frenkel, A. Klajman and I. R. Cohen. 1985. Arthritis induced by a T-lymphocyte clone that responds to *Mycobacterium tuberculosis* and to cartilage proteoglycans. *Proc. Natl. Acad. Sci. USA. 82:5117.*

5. Van Eden, W., J. E. R. Thole, R. van der Zee, A. Noordzij, J. D. A. van Embden, E. J. Hensen and I. R. Cohen. 1988. Cloning the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. *Nature 331:171.*

6. Billingham, M. E. J., S. Carney, R. Butler and M. J. Colston. 1990. A mycobacterial heat shock protein induces antigen-specific suppression of adjuvant arthritis, but is not itself arthritogenic. *J. Exp. Med. 171:339.*

7. Hogervorst, E. J. M., J. P. A. Wagenaar, C. J. P. Boog, R. van der Zee, J. D. A. van Embden and W. van Eden. 1992. Adjuvant arthritis and immunity to the mycobacterial 65kD heat shock protein. *Intern. Immunol. 4:719.*

8. Van den Broek, M. F., E. J. M. Hogervorst, M. C. J. van Bruggen, W. van Eden, R. van der Zee and W. B. van der Berg. 1989. Protection against streptococcal cell wall-induced arthritis by pretreatment with the 65-kD mycobacterial heat shock protein. *J. Exp. Med. 170:449.*

9. Ito, J., C. J. Krco, D. Yu, H. S. Luthra and C. S. David. 1991. Preadministration of a 65KDa heat shock protein, GroEL, inhibits collagen induced arthritis in mice. *J. Cell. Biochem. 15A:284.*

10. Thompson, S. J., G. A. W. Rook, R. J. Brealey, R. van der Zee and C. J. Elson. 1990. Autoimmune reactions to heat-shock proteins in pristane-induced arthritis. *Eur. J. Immunol. 20:2479.*

11. Jindal, S., A. K. Dubani, B. Singh C. B. Harley and R. S. Gupta. 1989. Primary structure of a human mitochondrial protein homologous to the bacterial and plant chaeronins and to the 65-kilodalton mycobacterial antigen. *Mol. Cell. Biol. 9:2279.*

12. Karlsson-Parra, A., K Soderstrom, M. Ferm, J. Ivanyi, R. Kiessling and L. Klareskog. 1990. Presence of human heat shock protein (hsp) in inflamed joints and subcutaneous nodules of RA patients. *Scand. J. Immunol. 31:283.*

13. Boog, C. J. P., E. R. de Graeff-Meeder, M. A. Lucassen, R. van der Zee, M. M. Voorhorst-Ogink, P. J. S. van Kooten, H. J. Geuze and W. van Eden. 1992. Two monoclonal antibodies generated against human hsp60 show reactivity with synovial membranes of patients with juvenile chronic arthritis. *J. Exp. Med. 175:1805.*

14. Munk, M. E., B. Schoel, S. Modrow, R. W. Karr, R. A. Young and S. H. E. Kaufmann. 1989. T lymphocytes from healthy individuals with specificity to self-epitopes shared by the mycobacterial and human 65- kilodalton heat shock protein. *J. Immunol. 143:2844.*

15. Lamb, J. R., V. Bal, P. Mendez-Samperio, A. Mehlert, A. So, J. Rothbard, S. Jindal, R. A. Young and D. B. Young. 1989. Stress proteins may provide a link between the immune response to infection and autoimmunity. *Intern. Immunol. 1:191.*

16. Anderton, S. M., R. van der Zee and J. A. Goodacre. 1993. Inflammation activates self hsp60-specific T cells. *Eur. J. Immunol. 23:33.*

17. Quayle, A. J., K. B. Wilson, S. G. Li, J. Kjeldsen-Kragh, F. Oftung, T. Shinnick, M. Sioud, O. Forre, J. D. Capra and J. B. Natvig. 1992. Peptide recognition, T cell receptor usage and HLA restriction elements of human heat-shock protein (hsp) 60 and mycobacterial 65-kDa hsp-reactive T cell clones from rheumatoid synovial fluid. *Eur. J. Immunol. 22:1315.*

18. Snippe, H., and C. H. Kaaieveld. 1989. The immunoadjuvant dimethyl dioctadecyl ammonium bromide. In: *Immunological adjuvants and vaccines, vol. 179.* G. Gregoriades, A. C. Allison, P. Post, eds. Plenum Press, New York. p.47.

19. Van der Zee, R., M. H. M. Wauben, T. H. A. Lots and W. van Eden. 1992. Simultaneous multiple peptide synthesis (SMPS) for the analysis of T cell epitopes. *J. Cell. Biochem. 16D:83.*

20. Shinnick, T. M. 1987. The 65-kilodalton antigen of *Mycobacterium tuberculosis. J. Bactertiol. 169:1080.*

21. Venner, T. J. and R. S. Gupta. 1990. Nucleotide sequence of rat hsp60 (chaperonin, GroEL homolog) cDNA. *Nucleic Acids Res. 18:5309.*

22. Hogervorst, E. J. M., C. J. P. Boog, J. P. A. Wagenaar, M. H. M. Wauben, R. van der Zee and W. van Eden. 1991. T cell reactivity to an epitope of the mycobacterial 65-kDa heat shock protein (hsp65) corresponds with arthritis susceptibility in rats and is regulated by hsp65-specific cellular responses. *Eur. J. Immunol. 21:1289.*

23. Rudensky, A. Y., P. Preston-Hurlburt, S. C. Hong, A. Barlow and C. A.Janeway. 1991. Sequence analysis of peptides bound to MHC class II molecules. *Nature. 353:622.*

24. Chicz, R. M., R. G. Urban, W. S. Lane, J. C. Gorga L. J. Stern, D. A. A. Vignali and J. L. Strominger. 1993. Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. *Nature. 358:764.*

25. Pope, R. M., R. M. Lovis and R. S. Gupta. 1992. Activation of synovial fluid T lymphocytes by 60-kD heat-shock proteins in patients with inflammatory synovitis. *Arthritis Rheum. 35:43.*

26. De Graeff-Meeder, E. R., R. van der Zee, G. T. Rijkers, H-J. Schuurman, W. Kuis, J. W. J. Bijlsma, B. J. M. Zegers and W. van Eden. 1991. Recognition of human 60 kD heat shock protein by mononuclear cells from patients with juvenile chronic arthritis. *Lancet. 337:1368.*

Description of the figures

Figure 1: Mt or hsp65 immunisation primes for T cell recognition of hsp65. Ten day PLNC from rats immunised with hsp65, Mt/IFA, or Mt/DDA were tested against hsp65, PPD and Mt over a range of concentrations (20 µg/ml, which gave maximal responses, shown here). Unimmunised splenocytes and PLNC from rats immunised with PBS/DDA were used as negative controls (PLNC from IFA immunised rats showed the same response pattern as PBS/DDA-PLNC). All S.E.M's were less than 20%.

Figure 2: Anti-MHC mAb inhibition of anti-hsp65 PLNC responses.
Ten day hsp65-PLNC were cultured with a range of hsp65 doses in the presence of 10 µg/ml mAb specific for RT1.B (OX6), RT1.D (OX17) and RT1.A (OX18). Anti-cmp = UD15 anti-chloramphenicol, isotype control antibody. All S.E.M's were less than 20%.

Figure 3: Responses to mycobacterial hsp65 peptides following immunisation with hsp65.
Responses to the entire panel of hsp65 peptides were analysed using ten day hsp65-PLNC (a), or an hsp65-specific T cell line (b), generated from the same hsp65-PLNC population. Peptides were tested at 5 and 20 µg/ml (20 µg/ml shown here). Responses to hsp65, PPD and Mt (20µg/ml) were all greater than 60,000 cpm in (a) and 150,000 cpm in (b). Peptides inducing significant responses are identified. Where overlapping peptides were stimulatory, the peptide giving stronger responses is identified. The results of this experiment were reproduced in three further experiments.

All S.E.M's were less than 20%.

Figure 4: Responses to mycobacterial hsp65 peptides following immunisation with whole Mt.
Responses to the entire panel of hsp65 peptides were analysed using ten day PLNC from rats immunised with Mt/DDA (a), or T cell lines generated from Mt/IFA-PLNC by restimulation with hsp65 or Mt (b). Peptides were tested at 5 and 20 µg/ml (20 µg/ml gave stronger responses, and is shown here). Responses to hsp65, PPD and Mt (20µg/ml) were all greater than 40,000 cpm in (a) and 150,000 cpm in (b). Peptides inducing significant responses are highlighted. All S.E.M's were less than 20%.

Figure 5: Response of T cell lines specific for defined hsp65 epitopes.
T cell lines were tested for responses to overlapping hsp65 peptides, corresponding rat hsp60 peptides, hsp65 and Mt over a range of Ag concentrations (10 µg/ml shown here). All lines responded to PPD and none responded to control hsp65 peptides containing other epitopes (data not shown). All S.E.M's were less than 20%.

Figure 6: Anti-MHC mAb inhibition of T cell line responses.
Epitope-specific and hsp65-specific T cell lines were cultured with irradiated APC and 1 µg/ml Ag (specific peptide for epitope-specific lines or hsp65 for the hsp65-specific line) in the presence of 10 µg/ml mAb specific for RT1.B (OX6), RT1.D (OX17) and RT1.A (OX18). Anti-cmp = UD15 anti-chloramphenicol, isotype control antibody. All S.E.M's were less than 20%.

Figure 7: Modulation of AA development by preimmunisation with hsp65 peptides.
Rats were immunized in the hind footpads with 100 µg of individual synthetic peptides or PBS in DDA seven days prior to AA induction using 5mg Mt in 100µl IFA injected i.d. at the base of the tail. Five rats were used in each preimmunization group. Arthritis scores were assessed daily from eight days after Mt injection.

Figure 8: PLNC responses of peptide-preimmunized AA rats.
Rats were preimmunizaed and Mt-immunized as described in Fig. 7. PLNC (pooled inguinal and popliteal LN) were isolated 42 days after Mt-immunization and tested for responses to hsp65 paptides containing defined

T cell epitopes (20µg/ml). All PLNC responded to hsp65, Mt (responses were all greater than 50,000 cpm). All SEM were less than 20%.

Figure 9: Modulation of AA using epitope-specific T cell lines.
Rats were administered with $5x10^6$ T cells i.v. in PBS or PBS alone at the time of AA induction using 5mg Mt in 100µl IFA injected i.d. at the base of the tail. Five rats were used in each group. Arthritis scores were assessed daily from eight days after Mt injection. Results using lines H.46 (specific for 226-235) and H.52 (specific for 256-265) are shown. Injection of lines H.36 and H.43 (specific for epitopes 180-188 and 216-225 respectively) had no significant effect on AA (data not shown).

Figure 10: Modulation of CP20961-induced arthritis by preimmunization with hsp65 peptides.
Rats were immunized in the hind footpads with 100 µg of individual synthetic peptides (211-225 or 256-270) or PBS in DDA seven days prior to AA induction using 2mg CP20961 in 100µl mineral oil injected i.d. at the base of the tail. Five rats were used in each preimmunization group. Arthritis scores were assessed daily from eight days after Mt injection.

Figure 11: Preimmunization with rat hsp60(256-270) does not protect against AA
Rats were immunized in the hind footpads with 100 µg of individual synthetic peptides or PBS in DDA seven days prior to AA induction using 5mg Mt in 100µl IFA injected i.d. at the base of the tail. Five rats were used in each preimmunization group. Arthritis scores were assessed daily from eight days after Mt injection. Preimmunization with a control peptide (hsp65 211-225) did not influence AA development (data not shown).

Figure 12.
Immunization with hsp65(256-270) and rat hsp60(256-270) primes for T cell responses to distinct epitopes. Rats were immunized in the hind footpads with either M.256-270 or R.256-270 in DDA. Seven days later PLNC were isolated and restimulated *in vitro* with the immunized peptide. The resulting T cell lines (line P.m52 recognizing M.256-270, and line P.r57 recognizing R.256-270) were tested for responses to mycobacterial and rat peptides in the presence of irradiated syngeneic spleen APC. All SEM were less than 20%.

4

Claims

1. Polypeptide containing a part of the amino acid sequence of the heat shock protein hsp65 of *Mycobacterium tuberculosis* as depicted in SEQ ID No. 1, comprising at least 5 aminoacids which are in the same relative position as the same aminoacids in a T cell epitope of hsp65 that contains at least 4 consecutive aminoacids which are identical with the corresponding mammalian hsp60 aminoacids.

2. Polypeptide according to claim 1, wherein the polypeptide comprises at least 5 aminoacids which are in the same relative position as the same aminoacids in one of the sequences 81-100 and 241-270 of SEQ ID No. 1.

3. Polypeptide according to claim 2, wherein the polypeptide comprises at least 5 aminoacids which are in the same relative position as the same aminoacids in one of the sequences 84-95 and 256-265 of SEQ ID No. 1.

4. Polypeptide according to any one of claims 1-3, wherein the polypeptide comprises 5-30 aminoacids of the amino acid sequence of hsp65.

5. Polypeptide analogue which exhibits the immunological properties of a peptide according to any one of claims 1-4, but which contains one or chemical modifications.

6. Nucleotide sequence encoding a polypeptide according to any one of claims 1-4.

7. Expression system capable of expressing a polypeptide according to any one of claims 1-4.

8. Microorganism containing an expression system according to claim 7.

9. Eukaryotic cell containing an expression system according to claim 7.

10. Cell expressing a receptor from a T cell activated by immunostimulation using a polypeptide according to any one of claims 1-5.

11. Antibody raised against a polypeptide according to any one of claims 1-5.

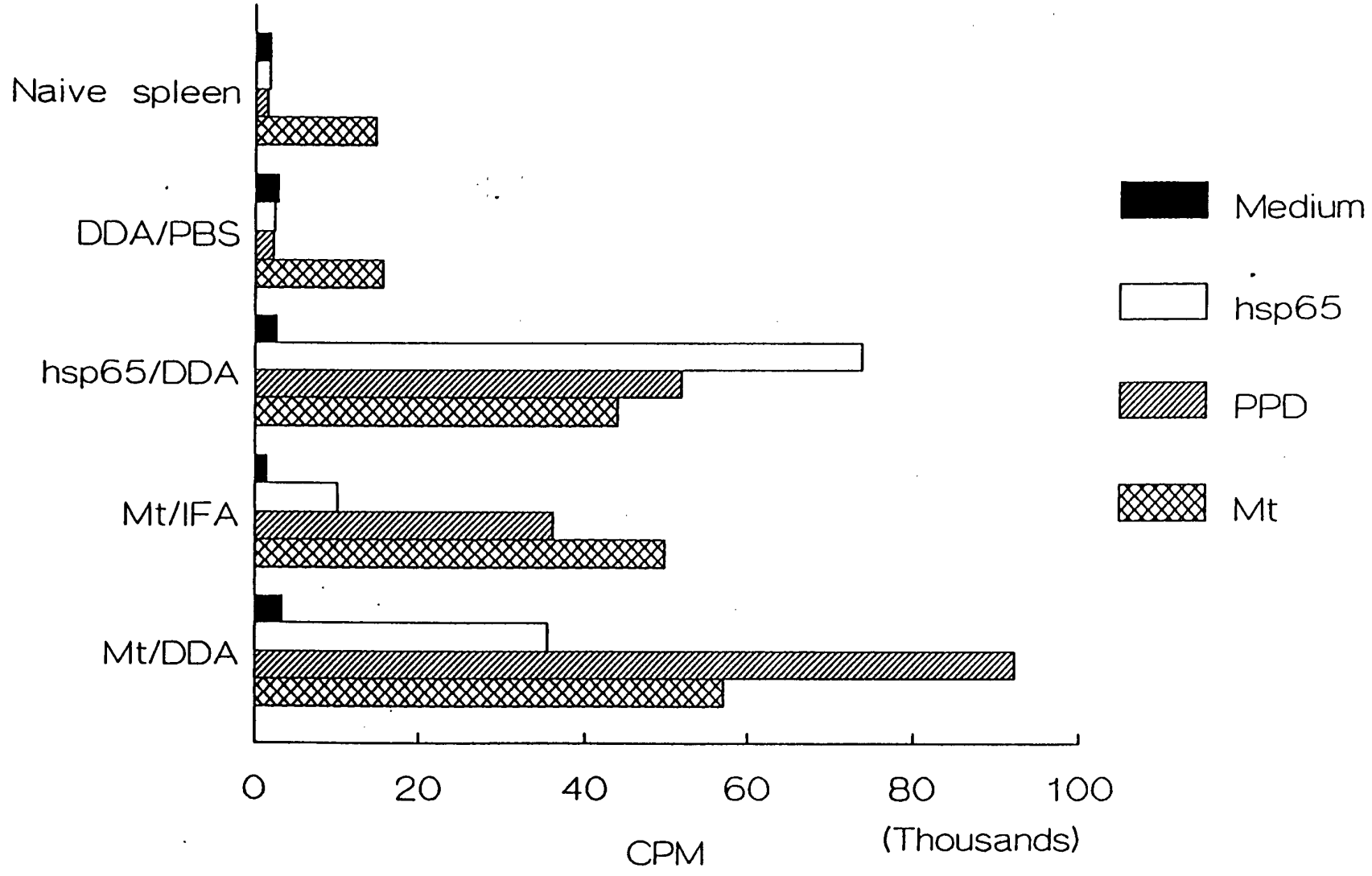
12. Pharmaceutical composition suitable for protection against or treatment of an inflammatory disease, including autoimmune diseases, diabetes, arthritide diseases, atherosclerosis, multiple sclerosis, myasthenia gravis, containing a polypeptide according to any one of claims 1-5, a nucleotide sequence according to claim 6, an expression system according to claim 7, a cell according to any one of claims 8-10, or an antibody according to claim 11.

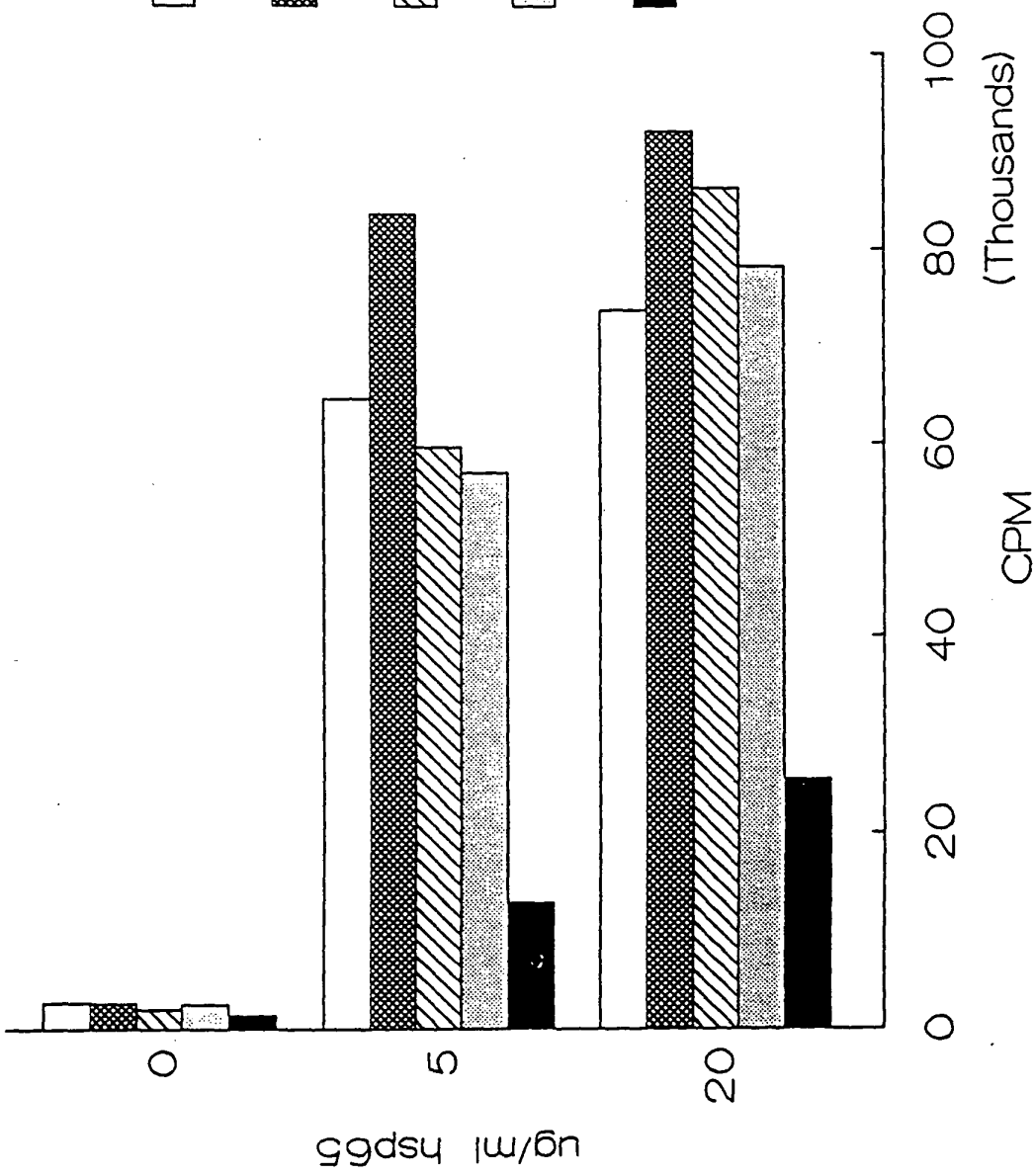
13. Diagnostic composition containing a polypeptide according to any one of claims 1-5 or an antibody according to claim 11.

Abstract

Polypeptides are provided which are useful for protection against or treatment of an inflammatory disease, including autoimmune diseases, diabetes, arthritide diseases, atherosclerosis, multiple sclerosis, myasthenia gravis. The polypeptides contain a part of the amino acid sequence of the heat shock protein hsp65 of *Mycobacterium tuberculosis* as depicted in SEQ ID No. 1, comprising at least 5 aminoacids which are in the same relative position as the same aminoacids in a T cell epitope of hsp65 that contains at least 4 consecutive aminoacids which are identical with the corresponding mammalian hsp60 aminoacids. Preferred T cell epitopes are located within the sequences 81-100 and 241-270 of SEQ ID No. 1.
Nucelotide sequences, expression systems, antibodies and pharmaceutical and diagnostic compositions derived from thee polypeptides are provided as well.

Figure 1


Naive spleen
DDA/PBS
hsp65/DDA
Mt/IFA
Mt/DDA
Medium
hsp65
PPD
Mt
0
20
40
60
80
100
CPM
(Thousands)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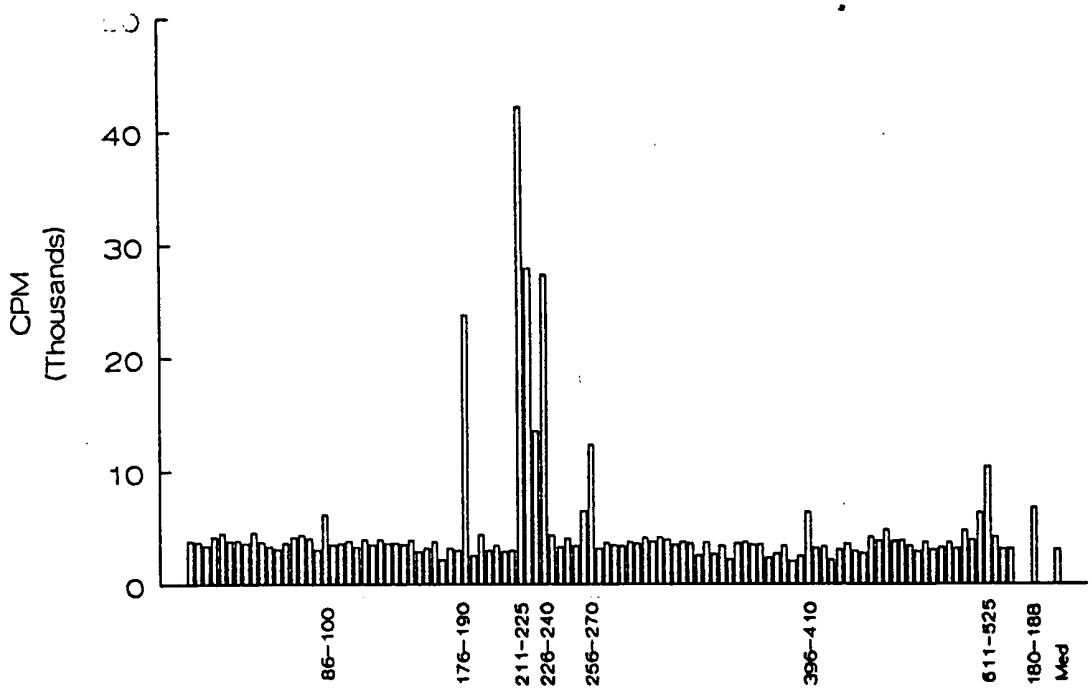
anti-cmp
anti-RT1.A
anti-RT1.D
anti-RT1.B
0
20
40
60
80
100
(Thousands)
CPM
0
5
20
ug/ml hsp65

Figure 2

Figure 3

A. Hsp65-PLNC



B. Hsp65-specific T cell line

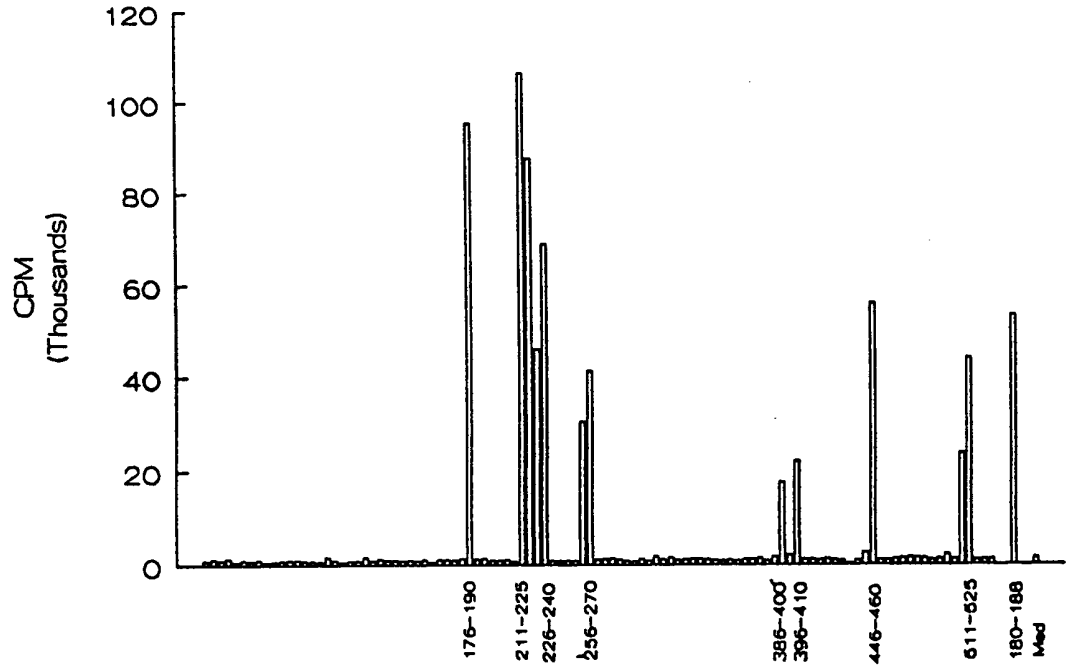
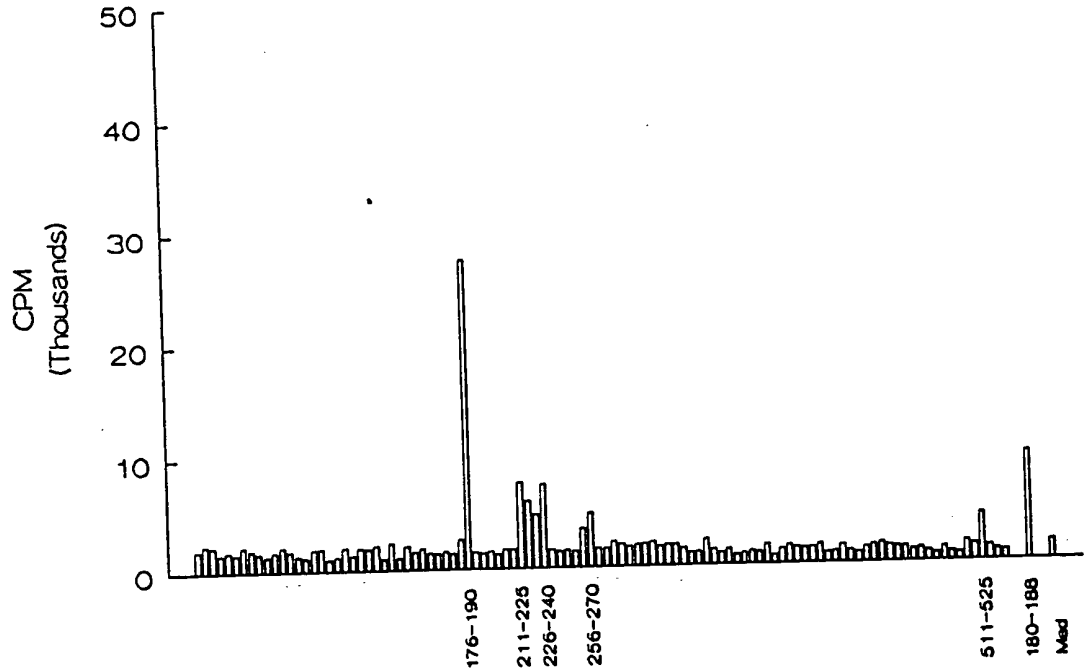
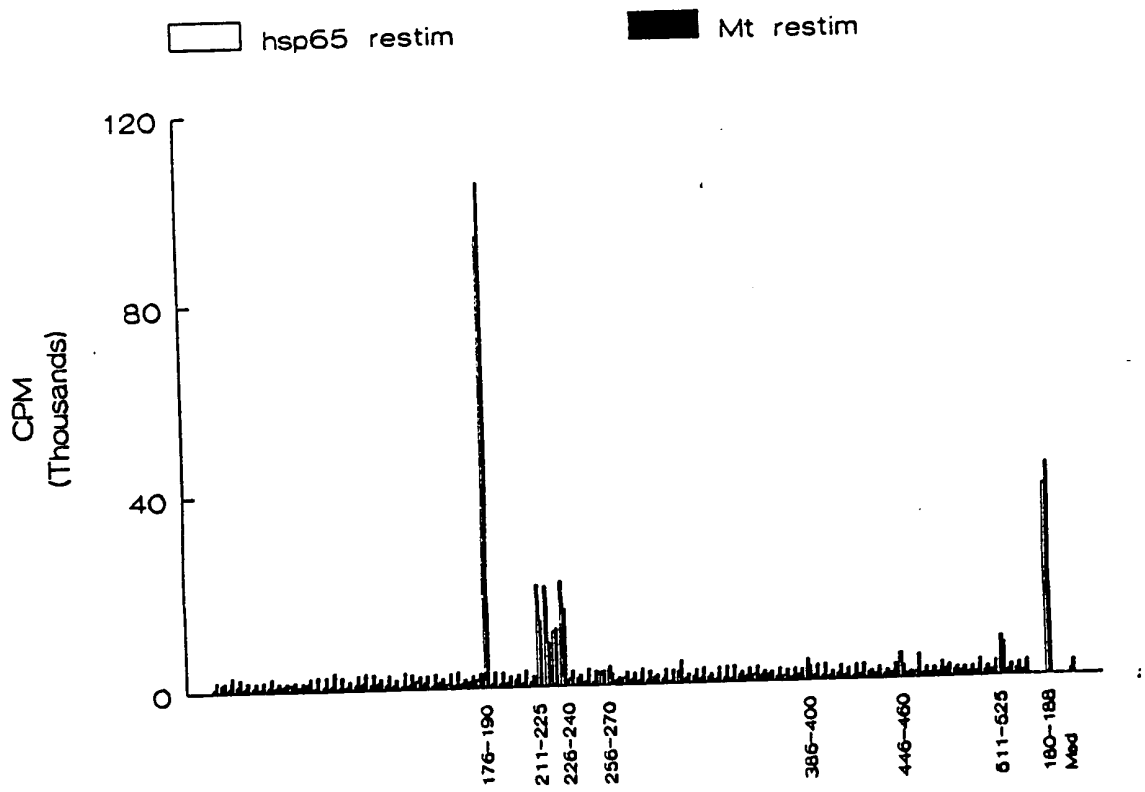


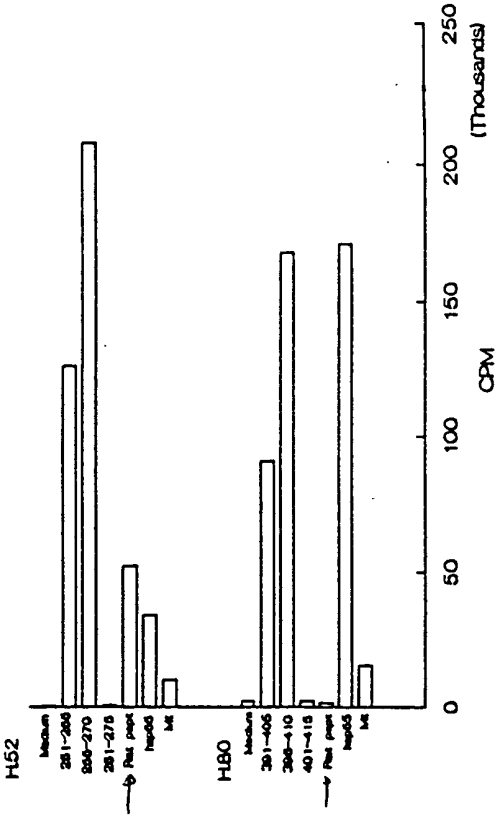
Figure 4

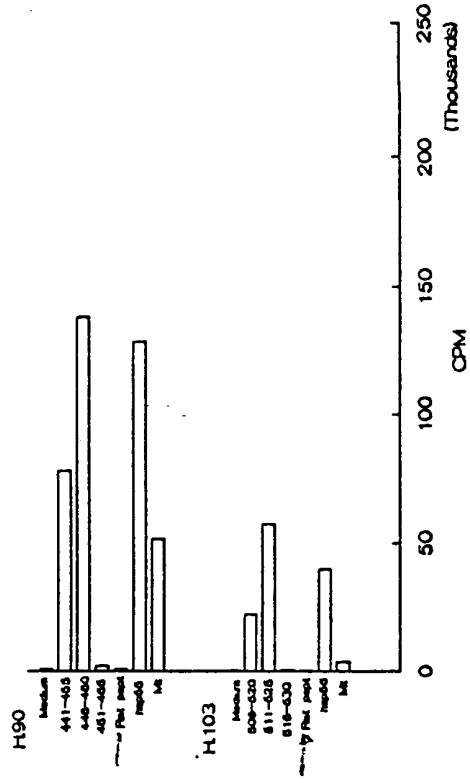
A. Mt/DDA-PLNC

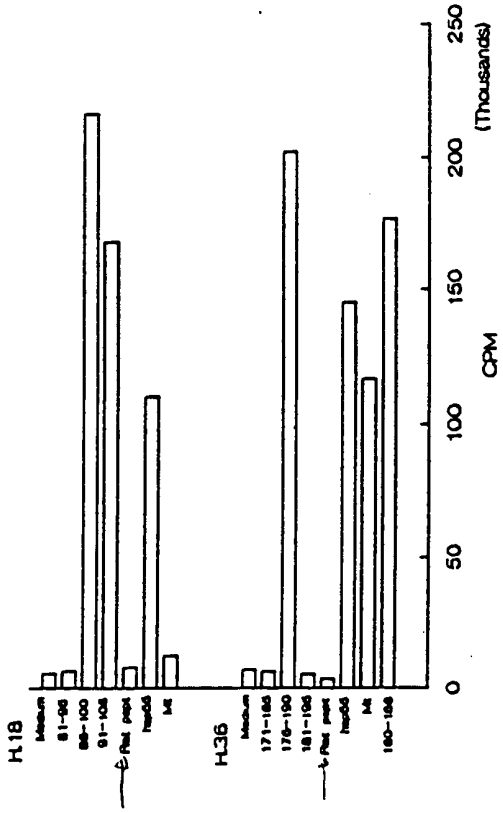


B. Mt/IFA-PLNC derived T cell lines




H52
H80
0
50
100
150
200
250
(Thousands)
CPM


H90
H103
0
50
100
150
200
250
(Thousands)
CPM


H18
H36
0
50
100
150
200
250
(Thousands)
CPM

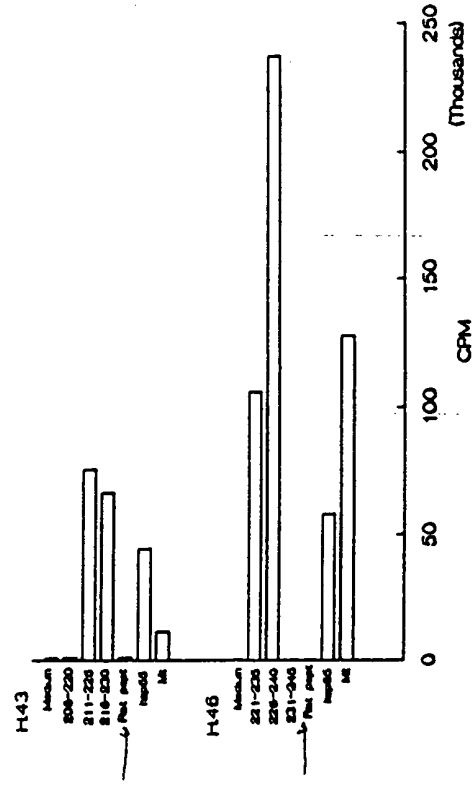

H43
H46
0
50
100
150
200
250
(Thousands)
CPM

Figure 5

Figure 6

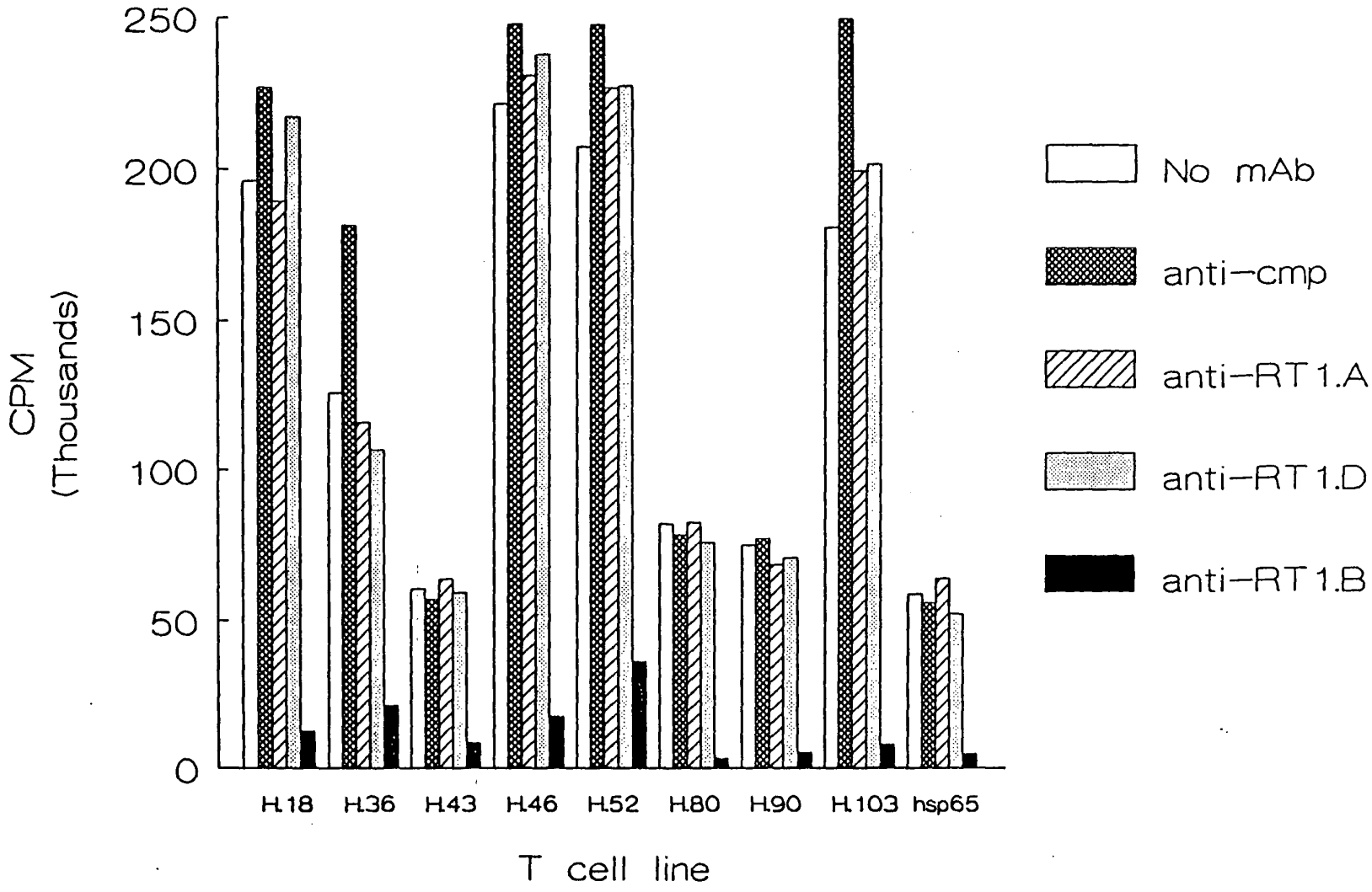

250
200
150
100
50
0
CPM
(Thousands)
H.18
H.36
H.43
H.46
H.52
H.80
H.90
H.103
hsp65
T cell line
No mAb
anti-cmp
anti-RT1.A
anti-RT1.D
anti-RT1.B

Figure 7

Modulation of AA by preimmunization with hsp65 peptides

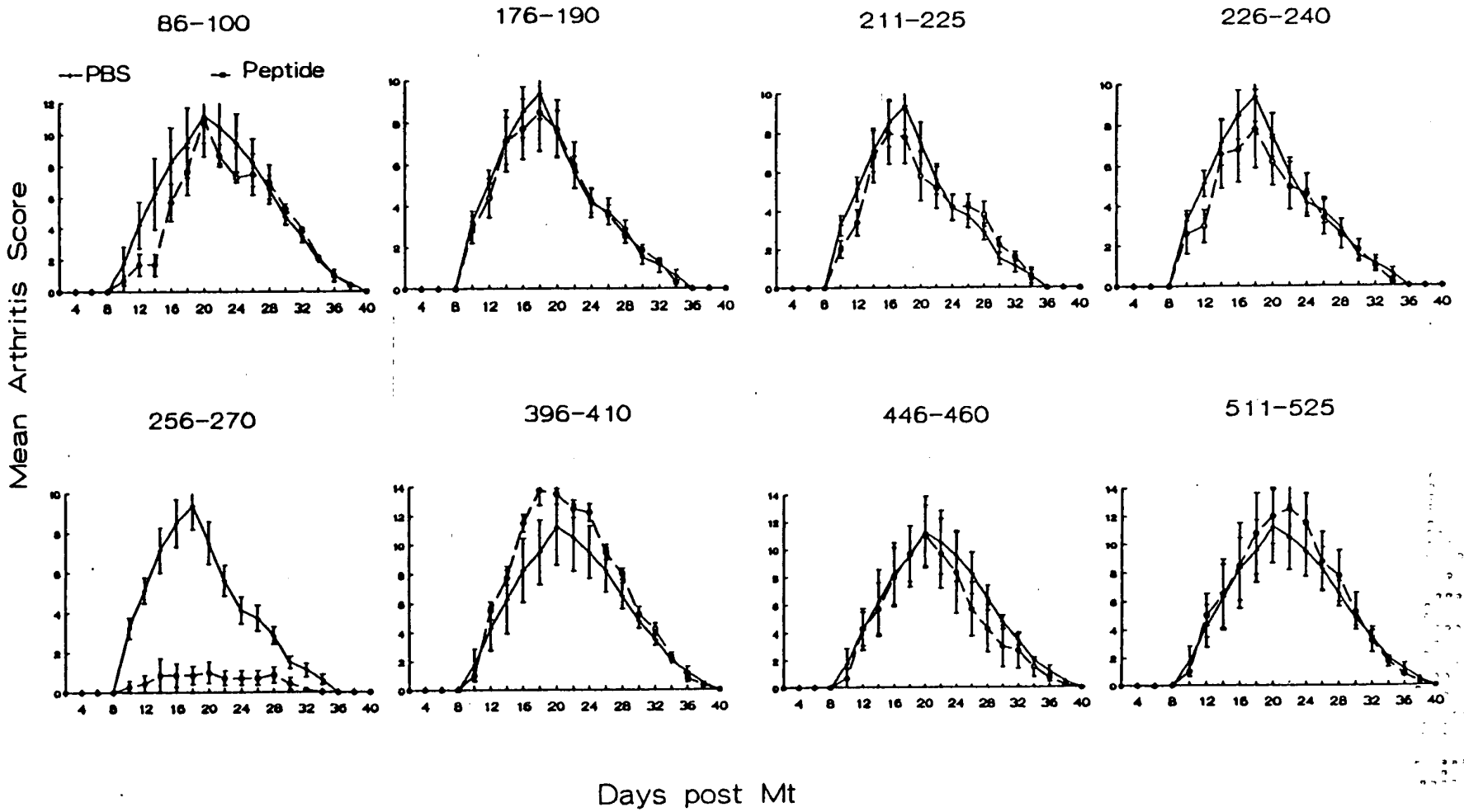
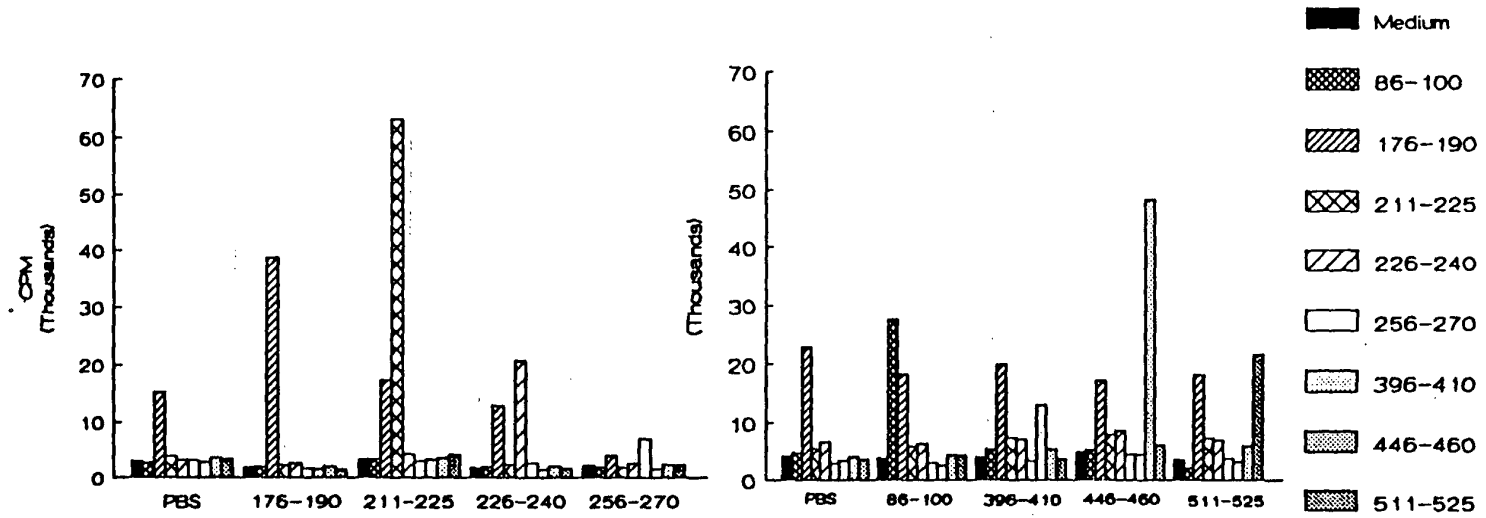


Figure 8

PLNC responses of peptide preimmunized rats



Preimmunizing peptide

Figure 9

Modulation of AA by T cell lines

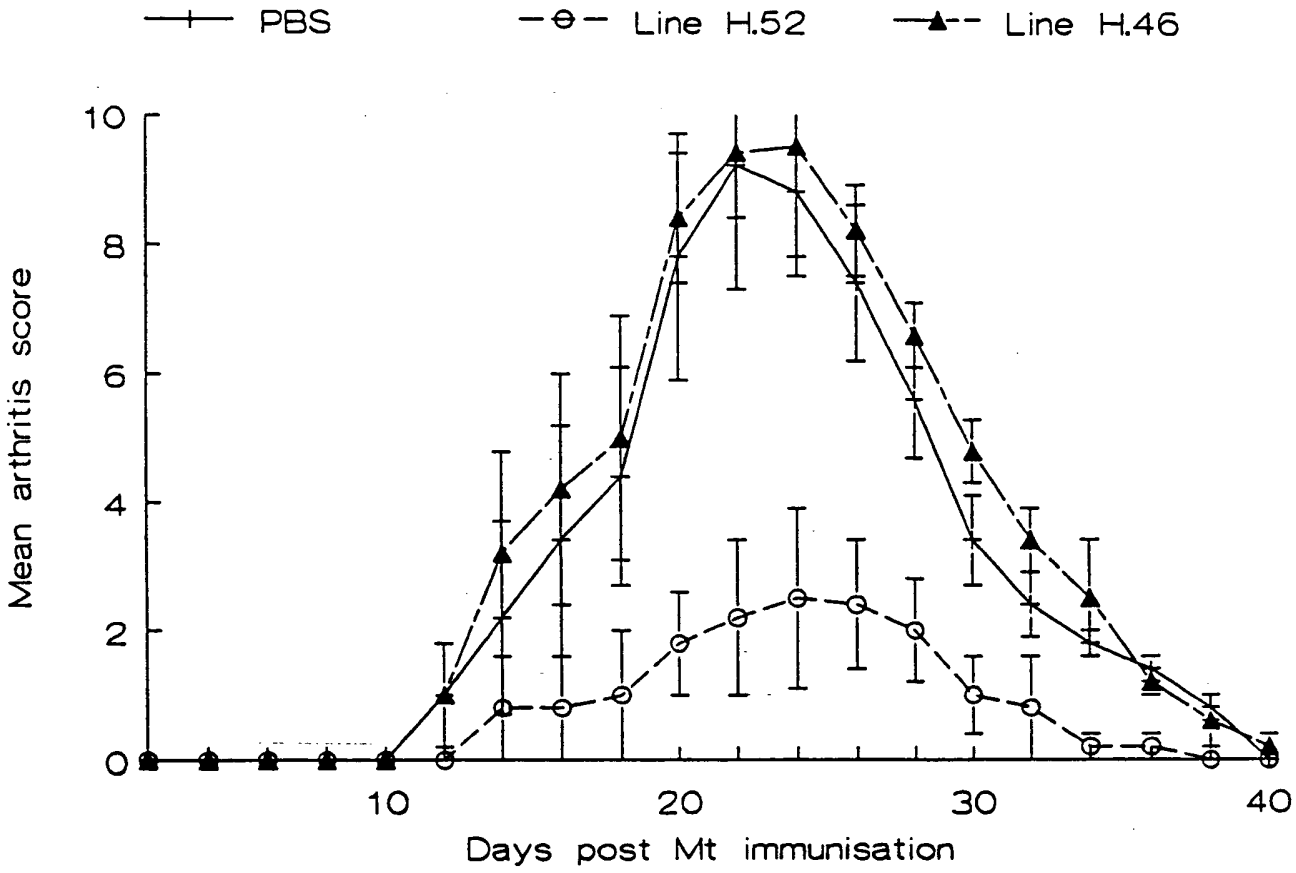


Figure 10

Modulation of CP20961-induced arthritis

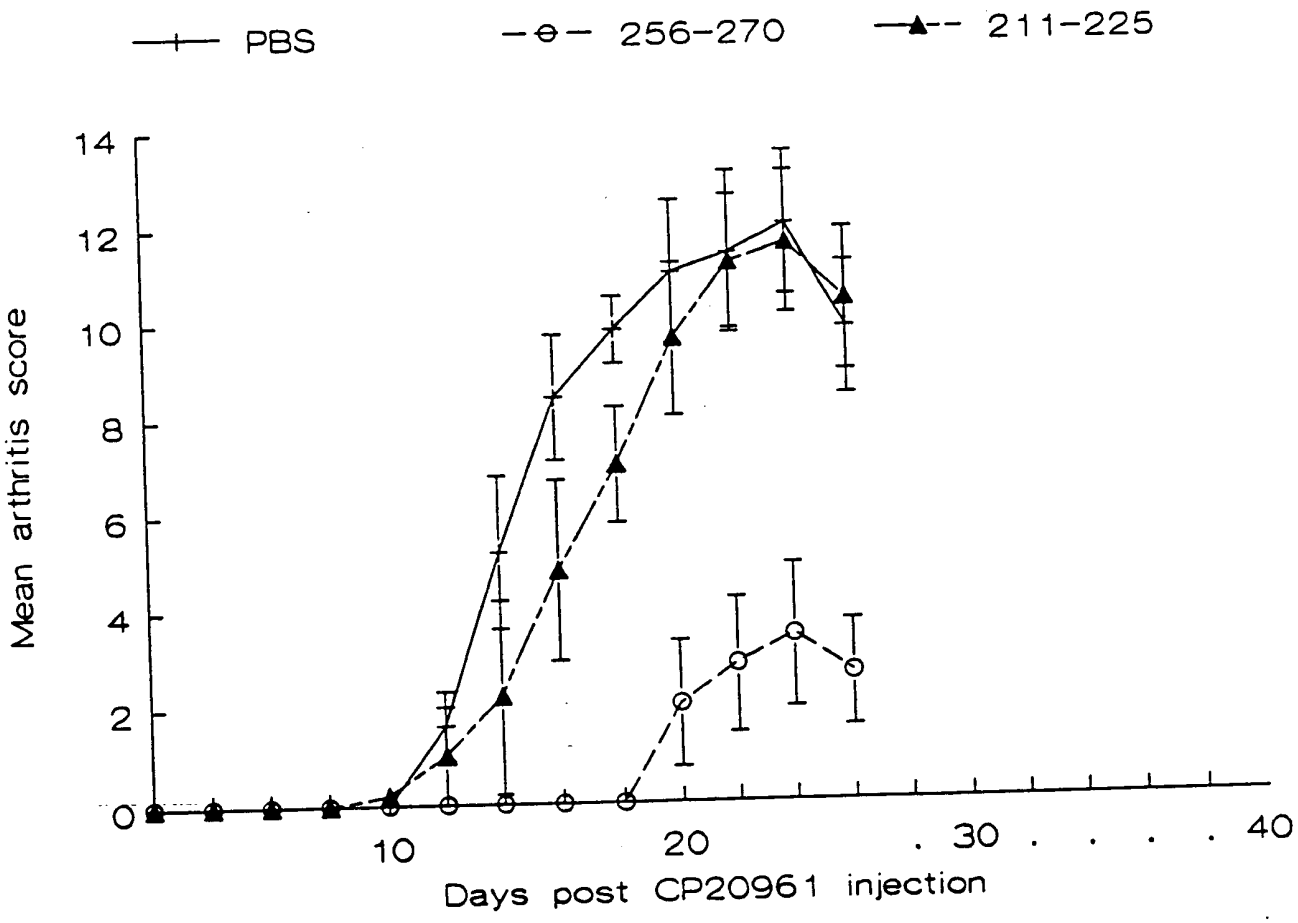


Figure 11

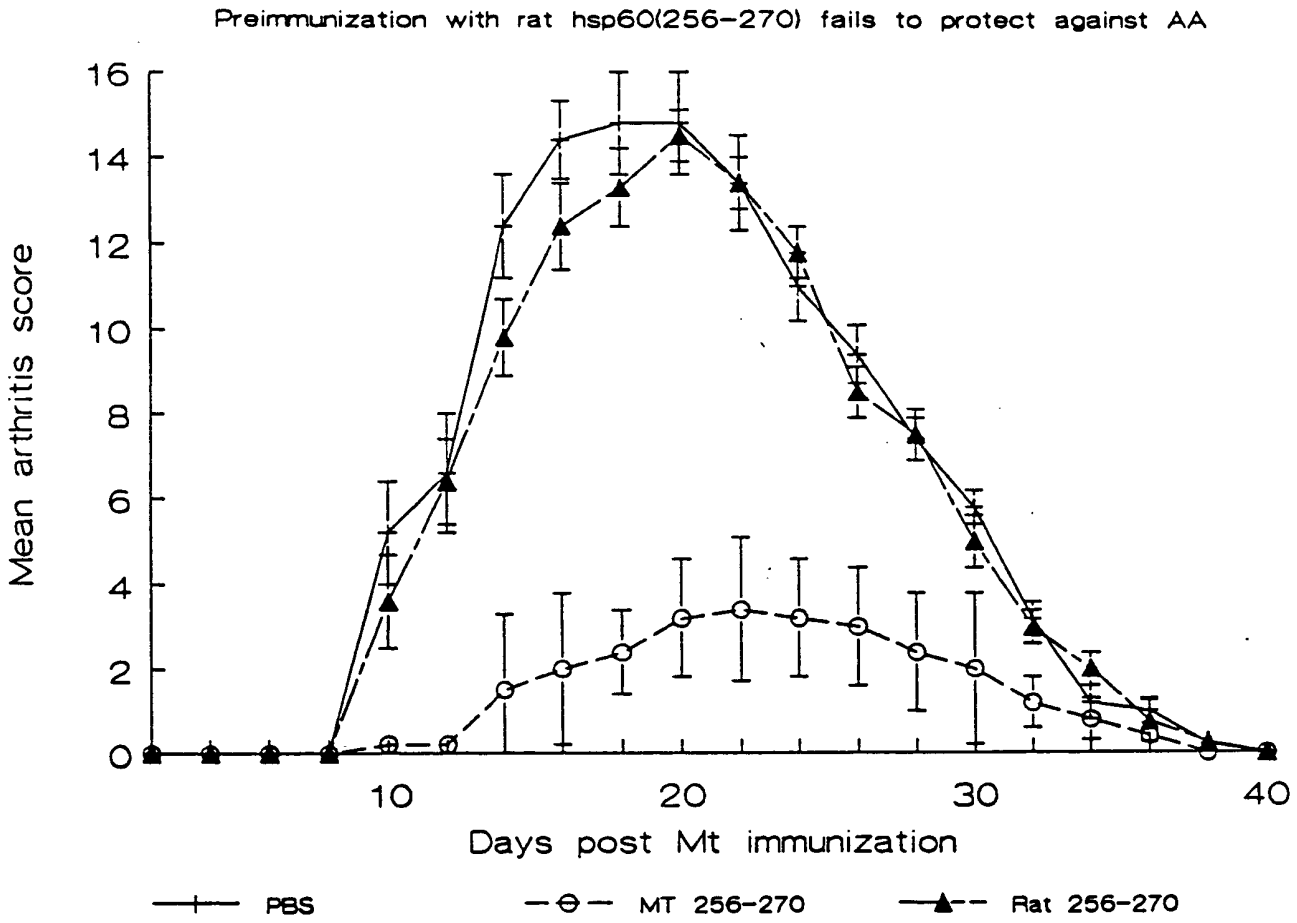

Preimmunization with rat hsp60(256-270) fails to protect against AA
Mean arthritis score
16
14
12
10
8
6
4
2
0
10
20
30
40
Days post Mt immunization
PBS
MT 256-270
Rat 256-270

Figure 12

Responses of T cell lines from rats immunized with mycobacterial or rat 256-270